

**Decennial Review Conference
on Cell Tissue
and Organ Culture**

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**Second Decennial Review
Conference on
Cell Tissue and Organ Culture**

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NATIONAL CANCER INSTITUTE MONOGRAPHS

KENNETH M. ENDICOTT, *Director, National Cancer Institute*

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ON
CELL TISSUE AND ORGAN CULTURE

THE TISSUE CULTURE ASSOCIATION

Held at

Bedford Springs Hotel
Bedford, Pennsylvania
September 11-15, 1966

Edited by
BENTON B. WESTFALL

**SECOND DECENNIAL REVIEW CONFERENCE
ON
CELL TISSUE AND ORGAN CULTURE**

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Address of Welcome

Virginia J. Evans
Co-Chairman of Conference

Ladies and Gentlemen:

In November, 1946, thirty-four scientists met in Hershey, Pennsylvania, to discuss the future of tissue or cell culture. They were the guests of the Committee on Growth of the National Research Council. At this historic first conference, the problems for which tissue culture might be of some value were brought into perspective.

Twenty-one of the participants who had had cell culture experience felt the Hershey conference had been so meaningful that there was need to form a permanent group to give impetus to this field of cell biology. Thus, the Tissue Culture Commission came into being. Soon the Commission became the Tissue Culture Association with many more members than the original 21.

Ten years after the conference, the First Decennial Review Conference was held in Woodstock, Vermont. There, in many fine formal papers and discussions, the past decade was reviewed and direction for the next 10 years was proposed.

As we embark on our Second Decennial Review Conference, we are most fortunate to have with us the chairmen of the two earlier conferences, Dr. Albert Claude and Dr. Philip White.

At the Woodstock conference, Dr. White spoke of the way in which these decennial conferences assist the individual and, may I add, the group to avoid both fruitless wandering in basically unrewarding cul-de-sacs and the equally undesirable neglect of valuable and unnoted trails to properly balance the search for new approaches with the development of details in already established ones and to maintain the equilibrium between theory and practice.

In this Conference on Cell Tissue and Organ Culture the same balanced objectives will be pursued. We hope to assess our problems from a broad, comprehensive point of view. This is not always possible in our annual conferences where some narrowness of vision is inevitable. Furthermore, we have chosen to have with us many participants from collateral fields to widen our horizons and sharpen our judgments. As we reason together, let us remember that we are from various disciplines. Let us avoid, as far as possible, the gobbledegook from the specialized languages of our respective fields.

Just before I left Washington, I found in our new *Washingtonian Magazine* an advertisement for a public relations company. It satirizes gobbledegook better than I can.

There was a young plumber who once discovered that hydrochloric acid was great for opening clogged pipes. He happily passed on his discovery to the Bureau of Standards.

The Bureau wrote to him hastily: "The efficacy of hydrochloric acid is indisputable, but the corrosive residue is incompatible with metallic permanence."

The plumber was delighted. He wrote to thank the Bureau.

The Bureau replied in even greater alarm: "We cannot assume responsibility for the production of toxic and noxious residue inevitable in the event of the employment of hydrochloric acid. Try an alternative."

Again the plumber wrote his pleased thanks.

This time the Bureau chiefs called in an information specialist who sent a telegram: "Don't use hydrochloric acid! It eats hell out of the pipes."

Here we are meeting face-to-face to discuss creatively in as plain talk as possible the limitations of techniques, theories, and results. The interchange of ideas that takes place will hopefully save some of us from carrying on research that seemed faultless at the time of first planning in the narrower confines of our laboratories and at our yearly sessions. To meet these objectives, we have visualized and planned this meeting as a working conference. It is the function of those who sit on the platform to inform us and stimulate the exploration of new fields. It is the function of those who sit in front of the platform to contribute to the growth of knowledge through pertinent comments and questions.

As we think together and plan our future research direction, it is imperative that we remember that while we have a future, we must review the past critically; we must also build upon that past research.

To carry on the working concept even further, the conference is being held at a resort with the expectation that additional knowledge and insight will be gained in the lobbies, on the golf course, on the tennis court, in the swimming pool, in the smoke-filled rooms, and in the bars.

I have been given to understand from the experience of others, of course, that John Barleycorn can strike sparks of genius in man and woman.

Before we settle down to, "burrow a while and build broad on the roots of things"—in the words of Robert Browning—there are other matters I should like to mention.

Support for this conference has come from three major sources:

The American Cancer Society has been generous in making it possible through grant-19 (1966) for the several session chairmen, the speakers, and panel discussants to be here.

The exhibitors have aided us in a multitude of ways.

Since no piece of work is complete until it is recorded and made available to all who may want to review the proceedings, we have planned to publish the record of this conference. The editors of the *Journal of the National Cancer Institute* have assumed this burden for us.

TABLE I		Summary of the results of the experiments	
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Introduction

Michael F. Dolan
Chairman, Program Committee

Ladies and Gentlemen :

The Second Decennial Review Conference on Cell Tissue and Organ Culture addresses itself to the question of the relationship between the cultured cell or tissues and its *in vivo* progenitor. It was felt that there is need to carefully define this relationship by review and by presentation of new data and the elicitation of incisive comments so that the role of tissue culture as a tool and as a discrete entity worthy of investigation in its own right might be more carefully evaluated.

The format of the Conference allows for a broadly comprehensive range in the particular field of tissue culture through maximum scientific exchange and relevant—and I stress “relevant”—speculation.

The sessions have been arranged in an attempt to allow for a logical development of the subject matter, starting with adaptive phenomena of cell populations through cell reactions and proceeding through cell interactions up to the question of the ultimate biological fate of cells after they have been in prolonged culture.

It is hoped that each session will set the stage for the next one and create a sense of anticipation in those assembled.

It should be understood that everyone attending this Conference is expected to contribute in some measure. The proceedings actually depend upon this for the fulfillment of their ultimate purpose. If there is maximum cooperation, there is good reason to anticipate that the directed efforts of the persons who will be seated upon the dias, supplemented by the challenging remarks of the people who are here as guests, will actually result in and assure that the goal of the Conference will be realized in its fullest measure.



NATURAL HISTORY OF *IN VITRO* ACCLIMATION AND THE INFLUENCE OF NUTRIENTS

Chairman: Professor E. Nevill Willmer, Cambridge University, Cambridge, England

Speakers: Dr. Charity Waymouth, The Jackson Laboratory, Bar Harbor, Maine
Dr. Harold Amos, Harvard Medical School, Boston, Massachusetts

Panelists: Dr. Kenneth S. McCarty, Duke University Medical Center, Durham,
North Carolina

Dr. Joseph F. Morgan, University of Saskatchewan, Saskatoon, Canada

Dr. Charles Wilde, University of Pennsylvania, Philadelphia,
Pennsylvania

Professor Willmer: The cytoplasm and the way a cell behaves can in essence be regarded as a complex physical-chemical system of proteins, lipids, and carbohydrates. Let us call that complex of proteins, lipids, and carbohydrates, "the complex." The activity of that system depends basically on two things. One is, that system will only be stable in connection with an environment, so we have the environment of the complex to consider. If we make any alterations in the environment, we shall certainly alter the complex. Similarly, any alteration in the complex will have repercussions on the immediate environment. So, we get a two-way reaction going on between the cytoplasmic or cellular complex and the environment.

On the other side, we have a very long and remarkable chain called the "genome." Let's call that "gene." Those in England will have that simplified, because we have the initials "G.C." and "E." as parts of this complex.

This system, and particularly the protein, depends entirely on what this pattern can produce. Similarly, this pattern is not all functional at the same time. Every cell in the body probably has far more information here than it ever uses at any one time. And the bit of information that is being used at the time will depend on what is happening here.

Therefore, the protein part of this is dictated by this system. And what this system does will dictate which parts of this are active. Thus it seems to me that the behavior of the cell complex depends on the interaction between the environmental side of the thing and the particular proteins being produced here that may modify this system.

It also seems that the discussion at this session really is based on an examination of the environmental system to determine how this will alter the activity of the cell.

Somatic Cells *In Vitro*: Their Relationship to Progenitive Cells and to Artificial Milieux^{1, 2}

CHARITY WAYMOUTH, *The Jackson Laboratory, Bar Harbor, Maine 04609*

SUMMARY

The relevance of the theme to the rest of the Symposium is outlined with emphasis on the complexity of the relationships between somatic cells *in vivo* and *in vitro*, and the programming of the dynamic processes of adaptation by which cells change, and are changed by, their environment. The nutritional requirements of cells for proliferation, function, and clonal growth are discussed in relation to the historical development of culture media. The interplay of genetic and environmental influences operating on explanted cells is demonstrated through a series of ques-

tions such as (a) What kinds of change do cells undergo in artificial milieux? (b) Are cells selected, mutated, modulated, differentiated, or dedifferentiated? (c) Which cell characteristics are stable or labile, progressive, and irreversible, or reversible, *in vitro*? (d) Is the loss of differentiated function an intrinsic loss of capacity to differentiate, or is its expression repressed by external factors? Most of these questions are elucidated through examples, though some of the answers are equivocal or inferential.—Nat Cancer Inst Monogr 26: 1–21, 1967.

HOW DO CELLS DIFFER FROM THEIR PROGENITORS?

Large parts of my subject—how cells in artificial media differ from or resemble their progenitors—belong in all the other sessions of this Conference. Let me illustrate this. (i) *Genetic constitution*: Every cell is the bearer of a genetic history. Its capabilities for differentiation and functional expression depend partly upon this history, and partly upon the environment. Harris (1964), in his book on somatic variations in cells, points out the important distinction between genetic and epigenetic mechanisms. True genetic mechanisms are those which “are concerned with the preservation and replication of information in structural form; for example, the molecular configurations of deoxyribonucleic acid or the

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

² Supported by a grant from The John A. Hartford Foundation, Inc.

individuality of self-duplicating cytoplasmic organelles. Epigenetic mechanisms, on the other hand, regulate the expression of genetic information. They serve to translate structural symbols into phenotypic reality." The genetic constitution of a cell, its constancy or variability, is therefore one of the most important characteristics to be considered as we study what happens to cells in an artificial environment.

(ii) *Enzymological equipment*: The way cells behave biochemically—their metabolism and functions—depends on their enzymological equipment as well as on available substrates, so our theme certainly belongs with taxonomic enzymology. (iii) *Differentiation*: Insofar as cells *in vitro* do differ from their progenitors, we cannot separate our discussion of these differences from questions of the meaning of the mechanisms controlling these processes. This aspect has been ably and extensively reviewed by Davidson (1964). (iv) *Cell interactions*: Moreover, the way cells behave *in vitro*, we know, is strongly conditioned by their interactions with similar, or dissimilar, cells in the environment. Hence our subject also belongs with the topic of cell interactions. (v) *Malignant transformation*: A striking and little understood characteristic of many cells in long-term culture is the transformation of normal into malignant cells. This we are also to hear about in another session. (vi) *Fine structure*: Undoubtedly, the changes we recognize at the microscopical or molecular levels have their counterparts in changes in fine structure, including chromosome changes. It might seem that there is little left for me to discuss.

The relationship of somatic cells *in vitro* to their *in vivo* progenitors is a complex one of many parameters. In order to cover some of the kinds of change that take place, I shall ask a number of questions and try to answer some of them through examples.

What kinds of change do cells undergo in artificial milieux? Among them we may identify morphological, antigenic, metabolic, and functional changes.

Are cells selected, mutated, modulated, differentiated, or dedifferentiated? Probably all occur.

When we study proliferation, do we select cells, or media, or both, which favor cell proliferation? Do our procedures dissociate the basic capacities of cells for proliferation from their functional capacities?

Do cells gain new properties or lose ones they had *in vivo*? Which characteristics are stable or labile, progressive and irreversible, or reversible, *in vitro*?

When differentiation fails to take place, *i.e.*, if visible or detectable differences are not manifested or recognized, is this due to loss of capacity for a particular expression or absence of an extrinsic stimulus to elicit a latent capacity?

DEVELOPMENT OF ARTIFICIAL MILIEUX

Our theme is bound up with the development of controlled, and more and more precisely defined, environments for explanted cells. As development progressed, the great sensitivity of cells to small changes in their external milieu became more and more apparent. Living cells are plastic, and are the loci of a multitude of molecular events and exchanges. These exchanges, we now know, are not confined to ions and small molecules, but include constant synthesis and turnover of macromolecules. We are aware, as we were not 10 years ago, that nucleic acids act as codes, messengers, and controllers of cell economy (Sirlin, 1963).

The history of cell nutrition has been reviewed many times (Stewart and Kirk, 1954; Eagle, 1955; Hanks, 1955; Biggers *et al.*, 1957; Morgan, 1958; Swim, 1959; Paul, 1960; Waymouth, 1954, 1960, 1964) so I will merely mention briefly a few of the points relevant to our theme. The first tissue culture media were biological fluids, chosen to imitate as closely as possible the natural environments of cells. One of the surprises uncovered by the use of sera and tissue extracts was the general lack of cross species reactions. In contrast to the incompatibilities of foreign sera or cells with intact animals, most cells in culture can thrive in serum from foreign species, *e.g.*, mouse cells grow in horse serum, or guinea-pig cells in calf serum, etc. Cells from two different species can not only thrive together in the same medium, but can form chimeric organs (Em. Wolff, 1964) and even interspecies hybrid cells (Harris, 1965), both *heterokaryons*, *i.e.*, cells with nuclei derived from different species, and *synkaryons*, cells with nuclei containing chromosomes from different species.

First attempts to replace complex biological media by chemically defined mixtures, by the Lewises (1912), were followed by those of Vogelaar and Erlichman (1933, 1936, 1937), and Baker (1936), Baker and Ebeling (1938, 1939). The attitude of these pioneers is exemplified by Baker's (1936) statement that her aim was to "synthetize an artificial medium which would prove adequate for the maintenance of cell life and multiplication." Developments in the science of biochemistry affected the design of media, so that as vitamins and hormones were identified and isolated, and amino acids purified, they replaced yeast or tissue extracts, peptones or blood digests, in new media. After Fischer's (1941, 1948) identification of amino acids as essential components of growth media, the first effective completely synthetic mixtures of White (1946, 1949); Morgan, Morton, and Parker (1950); Evans *et al.* (1956a and b); and Waymouth (1959) were constructed. Developments and refinements have made many others available today (Kitos, Sinclair, and Waymouth, 1962; Ham, 1963a; Nagle *et al.*, 1963; Liebovitz, 1963; Evans *et al.*, 1964; Waymouth, 1965).

CAPACITIES FOR PROLIFERATION AND FUNCTION

Almost all synthetic media were designed for long-term, progressive growth of cells. Because this approach was successful, *i.e.*, many kinds of cells would grow fast in similar media, it looked as if all cells had similar growth requirements (Levintow and Eagle, 1961). But this similarity is at least in part an artifact, for only those cells survived and grew that could use the particular combinations of components supplied to them. Moreover, different combinations may be equally effective, in view of such observations as that folic acid spares the need for glycine (Eagle, 1959), and that glycine, thymidine, and adenine spare the need for folic acid (Neuman and Tytell, 1960). Some cells have needs not exhibited by others. For example, Eagle *et al.* (1960) have grown cells with low ability to synthesize inositol in parabolic culture with strain L cells, which synthesize more than adequate amounts of this vitamin which is important for the utilization of purine bases and nucleosides (Tsukagoshi *et al.*, 1966). Many kinds of cells can successfully synthesize DNA, RNA, and the basic enzymes for continuing metabolism and proliferation, in synthetic media of simple basic composition containing salts, energy source, vitamins, amino acids, and a small number of other substances such as purines and pyrimidines. Such simple mixtures are also sufficient even for production of some specialized proteins, for example collagen and keratin.

Specific stimuli produce more specific effects on cells, *in vitro* as *in vivo*. Surprisingly, little attention was given to the hormones during the phase of great preoccupation with proliferation. Though Vogelaar, and Baker, designers of "imitative" fluids, did add insulin, thyroxin, "antuitrin," pitressin, etc., to their media, experience proved that media for rapid growth could dispense with this biologically important class of substances. They are, however, increasingly recognized as stimulants to special functions, and numerous examples of such effects have been documented in the decade under review (Lasnitzki, 1965).

REQUIREMENTS FOR CLONAL GROWTH

Those who grew cells in plasma clots and observed differences in cell behavior at varying distances from the explant realized that cell density affects survival and growth. There seemed to be optimal degrees of cell association for growth or differentiation. Groups of cells restrained from migration were more apt to undergo histotypic differentiation than cell division; by contrast, cells which grew out were regarded as "dedifferentiated" and restored to a more primitive state in which cell division was possible. This theory still persists, so it is instructive to recall some statements made over 40 years ago on the subject. "Growth," said Bur-

rows, "is not something peculiar to the younger cells. It is a reaction which may be the property of any fixed cell when placed in the proper environment. The environment suitable for such growth is one where the cells are crowded together and the circulation is reduced." (Burrows, 1923-24). "It has been found that stagnation and cell crowding are important for the growth of these cells because this growth depends on the accumulation about the cells of a certain concentration of a substance or substances formed by them. This substance or substances has been called *archusia* It can reach the concentration necessary for growth only when there are a sufficient number of cells forming it in a small quantity of medium from which it cannot escape." (Burrows, 1926).

The mystery of the failure of isolated, individual cells to divide was intriguing. It was solved, from the practical point of view, by application of Burrows' picturesquely described principle. Single-cell cloning was accomplished, first by Sanford (Sanford, Earle, and Likely, 1948; Sanford *et al.*, 1961a) by confining cells within narrow capillaries, with or without "conditioned" medium; and then by Puck, applying the same principle, by restricting diffusion (Puck, Marcus, and Cieciora, 1956), or by supplying another kind of conditioning from "feeder layers" of metabolizing but nonviable cells (Fisher and Puck, 1956). Single cells, or small inocula, require some components (no longer called *archusia*) that are apparently not needed by larger groups of cells in the same volume of medium. These are substances, as Burrows discerned, that cells synthesize too slowly to compensate for loss by dilution or removal from the cell. Media which, unsupplemented, are adequate for large cell populations, can be made useful for cloning single cells by adding compensating amounts of small molecules such as inositol (Eagle, 1956), serine (Lockart and Eagle, 1959), and others (Eagle and Piez, 1962; Dupree *et al.*, 1962; Sanford *et al.* (1963).

A long-disputed question has been whether macromolecules, especially proteins, are needed for the growth or clonal survival of cells. Several high-molecular-weight components useful for cloning have also been implicated as substances promoting adhesion of cells to glass or other substrates. Examples of dialysates replacing proteins suggest (Gwatkin, 1960) that proteins are not used *per se*, but after proteolysis. Macromolecular protein material loses activity on prolonged dialysis (Sato, Fisher, and Puck, 1957). Several specific protein fractions, both albumins and globulins (Puck, 1958; Lieberman and Ove, 1958), have been used to protect cells. One of the most popular of these has been the α_1 -glycoprotein fetuin (Fisher, Puck, and Sato, 1958, 1959; Lieberman and Ove, 1958), a protein with high anion-binding ability (Spiro, 1960), which, in the presence of Mg^{++} or Ca^{++} , was reported both to promote attachment to glass and to inhibit proteolysis. However, Lieberman, Lamy, and Ove (1959) found loss of activity of fetuin, as Dupree *et al.* (1962) found with

other proteins, when highly purified. The protamine salmine (Lieberman and Ove, 1958, 1959; Holmes and Wolfe, 1961); poly-L-lysine (Lieberman and Ove, 1958); and the anion-binding amine putrescine (Ham, 1964) promote cell adhesion. Linoleic acid can replace serum albumin in the presence of fetuin (Ham, 1963b). Chinese hamster cells have been cloned in defined media supplemented with thymidine, pyruvate, cysteine, asparagine, ferrous iron, and extra folic acid (Ham, 1960, 1965). Mouse cells are regularly cloned in defined medium by Sanford (personal communication). Cloning is possible in defined media, and proteins need not form part of cloning media.

Proteins are, however, definitely beneficial in otherwise deficient media, where they may act as carriers (Eagle, 1955; Eagle and Piez, 1960; Dupree *et al.*, 1962; Michl and Svobodova, 1965) of lipids, vitamins, trace metal ions, etc.; as sources of amino acids; or as detoxicants, binding injurious substances. Macromolecules other than proteins, such as methyl-cellulose, can carry out some of these protective functions (Kuchler *et al.*, 1960; Bryant *et al.*, 1961). Cloning is a rigorous test of an artificial milieu, and its practice has contributed to the identification of the components and environmental influences which single cells need, and enabled effective media to be designed (Paul, 1959; Neuman and Tytell, 1960; Sanford *et al.*, 1963; Ham, 1965). Empirically designed media are now sufficiently reliable and effective to use in the elucidation of questions about the relative roles of genetic and epigenetic mechanisms in maintaining stability, or promoting change, in cells *in vitro*. So we are ready to ask what role the cell itself plays in directing its own fate, and what role the environment, bearer of nutrients and stimuli, takes in determining the future of the cell.

SOME STABLE PROPERTIES OF EXPLANTED CELLS

Antigenic specificity is among the phenotypic characteristics which probably depend principally on genetic control and very little on environmental control. Although some kinds of antigenic specificity may be lost from cells after long cultivation (Weiler, 1959; Högman, 1960), species-specific and strain-specific antigens persist in cells grown for many years *in vitro* (Coombs *et al.*, 1961; Rosenau and Moon, 1961; Stulberg *et al.*, 1961; Sanford *et al.*, 1961b; Evans and Bryant, 1965), even in heterospecific or chemically defined media. Tissue-specific antigens also persist. Human cells, after many years in cultivation, retain esterases with specific electrophoretic patterns corresponding to those of fresh human tissues (Paul, 1959; Komma, 1963).

NUCLEAR CONTROL MECHANISMS

The classical experiments of Briggs and King (1952) on nuclear transfer opened a new field of research on the effects of different cytoplasmic environments on the nucleus and vice versa. Recently, Harris and Watkins (1965) added to our understanding of the relative roles of nucleus and cytoplasm in cells in culture. Human and mouse tumor cells in mixed suspensions were hybridized by treatment with an ultraviolet-inactivated virus. Harris (1965) followed this report by one on the fusion of HeLa (human tumor) cells with highly differentiated normal cells from three species—rat, rabbit, and fowl. These were short-term experiments, with important implications in delineating the roles of nucleus and cytoplasm (which in turn is affected by the medium) in cell cultures of longer duration. Harris' heterokaryons show greater activity in RNA synthesis than the differentiated cells contributing nuclei to the hybrid cells. Still more striking is the effect on DNA synthesis. Nuclei, even from fowl erythrocytes, in which DNA synthesis is zero, "redifferentiate" or at any rate regain, in the environment of another cytoplasm, the capacity to become sites of DNA synthesis. We may infer that nuclei from these highly differentiated cells have not lost irreversibly the ability to take part in DNA and RNA synthesis.

Nuclear control of the rate of synthesis of characteristic cell products is strongly suggested by the elegant studies of Green *et al.* (1966) on rates of collagen and hyaluronic acid synthesis in hybrid cells. A mouse cell line, 3T6, clone 7, with high rates of collagen and hyaluronic acid synthesis, was hybridized with each of two other mouse cell lines—NCTC 2555, and NCTC 2472, clone 6—both of which produce little collagen and no hyaluronic acid. Clones of the hybrid cells, recognized by marker chromosomes from each parent line, produced collagen and hyaluronic acid at rates intermediate between those of the parent lines.

DIFFERENTIATION: LATENT OR LOST?

There are many examples of dividing cells that do not differentiate, in apparent support of the old theory that cell division and cell differentiation are incompatible. But the theory has proved to be an oversimplification (Grobstein, 1959; Davidson, 1964). Resseguie (1961), for example, described conditions compatible with myogenesis which favor proliferation of chick embryo cells. A heteroploid line of rat cells from the rat eye (ARE-2-60) actively produces acid mucopolysaccharides under conditions favorable for proliferation (Davidson, 1963, 1964). But more generally, cells either divide or differentiate, not both at once, though cessation of

DNA synthesis does not necessarily mean that this function cannot be resumed (Stockdale and Holtzer, 1961) as Harris' (1965) experiments demonstrate. Holtzer's (1961, 1963) results with myoblasts *in vitro* support the thesis that division and differentiation are usually not concurrent. He demonstrated by isotopic labeling that dividing myoblasts produce no myosin, while myosin-producing cells neither synthesize DNA nor divide. Holtzer raises the highly important question whether DNA synthesis is blocked when myosin synthesis begins, or whether DNA synthesis can begin only after myosin synthesis has been shut off. Adams *et al.* (1965) have helped solve this kind of question by investigating whether initiation of DNA synthesis promotes the production of DNA polymerase, or whether DNA polymerase activity increases before DNA synthesis can occur. In adult rabbit kidney cells freshly isolated in culture, it appears that the enzyme must be available before DNA synthesis and cell division can begin. However, this cannot be the only mechanism controlling DNA synthesis, because in continuously proliferating cell lines such as strain L, the level of DNA polymerase is constant and not related to the cycle of DNA synthesis. It may, however, be a question, not of enzyme synthesis, but of conversion from an inactive to an active form, as Littlefield *et al.* (1963) have demonstrated does occur in L cells. Extrinsic factors such as levels of energy sources and substrates may control the turning on and off of DNA synthesis.

There are now many examples of the effects of extrinsic factors on expression of differentiated functions *in vitro*. Among these we may select the work of Konigsberg and Hauschka (Konigsberg, 1963; Konigsberg and Hauschka, 1965; Hauschka and Konigsberg, 1966). Embryonic chick myoblasts were grown to confluency in monolayers without displaying any evidence of differentiated function. Under appropriate environmental stimulus, these cells became multinucleate and synthesized contractile proteins. This differentiation of myoblasts into muscle occurred either in proximity to fibroblasts, or when the cells were exposed to "conditioned" medium. The common factor was found to be the presence of collagen, which itself stimulated muscle differentiation from the apparently undifferentiated cells.

Cartilage cells, when stimulated to divide, lose, within 4 or 5 division cycles, the ability to produce chondroitin sulfate. Holtzer *et al.* (1960), who grew these cells under conditions which favored cell multiplication, and then put them into organ culture in the form of compact pellets, found that such cultures failed to behave like chondrocytes; *i.e.*, unlike fresh cells grown as pellets, they did not synthesize chondroitin sulfate. These results led Holtzer *et al.* to ask, but not to answer, several provocative questions:

"Does a given chondrocyte dedifferentiate gradually or abruptly? How dependent upon mitotic divisions is the process of dedifferentiation? Do de-

differentiated chondrocytes continue to synthesize collagen or the protein associated with chondroitin? Can dedifferentiated chondrocytes differentiate into other types of cells or can they only differentiate into chondrocytes?" These questions raise important and pertinent issues and await definitive answers.

A related question is, To what extent is the differentiation of various stem cells genetically controlled and predetermined, and to what extent dependent upon a proper environment (Leblond, 1964)? Holtzer (1963) observed that presumptive chick myoblasts, after 4 to 6 divisions *in vitro*, cease to form myoblasts, just as chick chondrocytes, after a similar period in culture, cease to be chondrocytic (Holtzer *et al.*, 1958). These cells give rise to cells not producing the characteristic molecules, myosin or chondroitin. We do not know whether we should call this dedifferentiation, or repression of latent capacities for differentiation not expressed under a particular set of conditions *in vitro*. We do know that, as exemplified by the experiments of Harary *et al.* (1966), the presence of particular molecules in the medium can elicit the expression of differentiated function. Nonbeating heart cells, grown in lipid-free medium, resume beating when lipids, specifically the fatty acids linoleic and palmitic acid, are supplied. This effect might be partly due to restoration of lipid as an energy source, but Harary's hypothesis is that lipid may be important for membrane integrity and functions.

In the adrenal tumors cultivated by Sato *et al.* (Sato *et al.*, 1964, 1965; Yasumura *et al.*, 1966), the cells' dependence on nutritional environment for their ability to express a differentiated characteristic was demonstrated. Two kinds of cells were cloned from the tumor cultures. The fibroblasts, which would overgrow the epithelial cells in uncloned cultures, have none of the characteristic properties of adrenal cells. The epithelial cells maintained their functional characteristics for over 100 passages *in vitro*. In a high-serum medium, they secrete steroid hormones. In low-serum medium, the ability to secrete hormones is lost, but can be restored on transfer to a high-serum medium. The epithelial tumor cells respond specifically to adrenocorticotropin (ACTH) (Yasumura *et al.*, 1966). The genetic capacity to produce hormones evidently remains latent in these cells until it is elicited by the proper stimulus. A most interesting example of redifferentiation in a suitable milieu is described by Main (1966). Mouse tooth germ cells, after growth for several weeks in unorganized culture, when transplanted to the base of the tail of isologous mice, form near-perfect teeth.

The capacities of a cell in culture for growth, for differentiation, or for function may be elicited or repressed, remain latent and be evoked again, by appropriate molecular stimuli. In differentiation, some pathways are shunted from synthesis for cell replication to synthesis for function, but not necessarily irreversibly.

PROGRESSIVE, IRREVERSIBLE CHANGES IN CELLS *IN VITRO*

Hayflick and Moorhead (Hayflick and Moorhead, 1961; Saksela and Moorhead, 1963; Hayflick, 1965) described human cell lines which remain diploid for 50 ± 10 cell replications, and then either cease dividing, or transform into viable heteroploid cells with different properties from the parent cells. These, as well as cells which undergo malignant transformation *in vitro*, seem to be irreversibly altered. Is the failure of human cells to continue to grow due, as Hayflick implies, to an inherent cell aging associated with a genetic incapacity for further divisions? Or can failure be attributed to inadequacies in the environment? Todaro and Green (1963) suggest that, in establishing cell lines from mouse embryonic cells, changes in cell properties occur which enable them to grow progressively, but that the change from diploidy to aneuploidy follows, rather than precedes, increase in growth rate. Established cell lines from animals of other species, *e.g.*, hamster (Yerganian and Leonard, 1961) can remain diploid. Clarification of this issue requires more information on genetic versus epigenetic factors in loss of diploidy and ability to grow progressively. The failure of human diploid cells may be genetically controlled, but a variety of epigenetic agents, from irradiation to viruses, have been definitely identified and implicated in malignant transformation, and may also be involved in some heteroploid transformations *in vitro*.

EPIGENETIC CONTROL MECHANISMS

The common practice has been to use basic media, similar in chemical composition, for cells of various origins, and to expect cells with different genetic endowments to express differences within such a uniform environment. We should now pay more attention to the use of specific keys to unlock specific capacities of cultured cells. Organ culture work has given many leads, and examples are not wanting, such as the effect of lipids on heart beat quoted before (Harary *et al.*, 1966), from cell culture literature (Eagle, 1965). For example, the classic work of Fell and Mellanby (Fell and Mellanby, 1953; Fell, 1957, 1961) demonstrated the very specific effect of vitamin A in eliciting the capacity of keratinizing epithelium to shift to synthesis of mucopolysaccharides. On the other hand, Wessells (1961) showed that methionine is necessary for initiation of keratinization of chick embryo skin. Sidman and Pearlstein (1965) identified a single amino acid which triggers a specific synthetic process. Mice of the pink-eyed dilution (p/p) or pink-eyed dilute (p^1/p^1) genotypes produce no retinal pigment *in vivo*. Retinae from these animals also fail to synthesize melanin *in vitro*, in a medium lacking tyrosine, but do so in its presence. This locates the defect in an inability of tyrosine *in vivo* to reach the reaction site, but shows that the retinal cells have a latent capacity to synthesize

melanin, not expressed *in vivo*. Another way to control pigment formation *in vitro* is illustrated by the work of Cleffmann (1963), in whose system the —SH concentration in the medium controls the formation of pigment by melanocytes from agouti mice.

There are many examples of epigenetic control exercised through feedback mechanisms by nutrients in the medium. One recent report will exemplify this. Bailey (1966) showed that the synthesis of lipid from glucose or acetate is inhibited by serum, or by certain lipids, including cholesterol. Over 20% of all lipid synthesis in L cells in synthetic medium NCTC 109 is from acetate. This synthesis is almost completely repressed in serum-containing media. Over 50% of the lipids are derived from glucose in NCTC 109. This synthesis is reduced by about 70% in serum- or lipid-containing media.

CONCLUSION: THE RANGE OF CHANGE

Somatic cells, *in vitro* as *in vivo*, change and are changed by their environment. This is a dynamic process of adaptation, sensitive to very delicate signals. Adaptation works through the genetic information system of the cell, which is itself subject both to mild adaptations; for example, in enzyme repression and derepression, induction and feedback inhibition; and to drastic transformations, culminating in the gross chromosomal aberrations often seen in the heteroploid cells which best survive in man-made environments. Heteroploid cells have, *ipso facto*, a genetic imbalance, which may be expressed in loss of old or gain of new phenotypic characteristics, including malignant transformation. The past decade has given us a new perspective on the relationships of cells to their environment. Perhaps in the next, full control of cell differentiation and function will be achieved *in vitro*, by the creation of environments which allow a single stem cell *in vitro* to be followed, from early undifferentiated stages, through each normal phase of cell division and modification of synthetic abilities, to a fully differentiated, functional cell.

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DISCUSSION

Dr. Morgan: The function of the panelists, as stressed by the organizing committee, has been to make comments, add observations, and particularly to ask penetrating questions. Unfortunately, Dr. Waymouth has made it very difficult by asking herself more penetrating questions than any I have been able to prepare. From her presentation, we have a tremendous body of information on cell nutrition generally.

As the work of Dr. Evans, Dr. Earle, and the Bethesda group has shown, it has been possible for years to grow many lines of cells in completely defined medium.

With other types of cells, it is sometimes necessary to supplement the chemically defined medium with various types of sera.

This raises a question which Dr. Waymouth posed: What is the exact role of the serum in tissue culture media? Does the serum serve a role in itself or does it serve as an inactive carrier for active small molecules? Does the serum in fact penetrate the cell as an entity, or must it be broken down by proteolytic enzymes to form active small molecules which then penetrate the cell? These are areas where further work is certainly required.

The role of peptides in cell nutrition has never been clearly delineated. When substances such as lactalbumin hydrolysate are added to chemically defined media that appear to have adequate amounts of amino acids or vitamins, they still cause a marked stimulation of many types of cultures.

Again, are we dealing here with active small molecules? Are we dealing with peptides that appear from some experiments to be in the range of 15-20 amino acid residues? Are substances of this nature related to the types of materials that Dr. Amos will discuss?

Another unclear area in cell nutrition work is that of the role of many cofactors in the culture environment. A number of years ago, while investigating the carbohydrate requirement of chick-embryo heart cells, Dr. Morton and I found that many carbohydrates would, indeed, serve as the sole energy source to maintain survival of these cells.

One notable exception was the compound hexose diphosphate of the Embden-Meyerhof pathway, a compound shown earlier by Albert Fischer to be extremely important in cell nutrition. When we investigated this anomaly further, we found that if we added quite high levels of adenosine diphosphate to our medium 199, which already contained adenosine triphosphate, the cells suddenly had the ability to utilize hexose diphosphate as the sole source of energy. The point is that we are dealing, not with a simple requirement, but with what appears to be an associated requirement. In fact, we are dealing with a balance in the culture medium, a balance between a phosphate donor and a phosphate acceptor.

The question, then, is: Are other balances necessary in our culture medium? Can failure to obtain propagation of some types of cells in a chemically defined medium be due to the inadequacy of the medium, or can it be possibly attributed to a defective balance between certain nutrients and certain groups of nutrients? These and many other questions still remain unanswered.

There are similar problems in the nutrition of the tumor cell and the whole question, again, of whether or not major differences exist between normal and malignant cells, differences which can be the basis for devising a form of chemotherapy.

One promising area here, which should be stressed, is the observation we reported some years ago that malignant cells in culture removed glutamine almost completely from the surrounding medium, while fresh, normal tissues actually increased the amount of this metabolite in their environment. Further work on this point is indicated.

Most studies on cell nutrition have been aimed at developing systems for the rapid and continuous propagation of many types of cells. In our preoccupation with this endeavor, it might legitimately be asked whether we are not, in fact, concentrating on developing an unphysiological system. Are we neglecting primary cultures and the information that can best be obtained during that initial stage between the *in vivo* and *in vitro* systems?

Dr. Waymouth: I will comment at least on one of Dr. Morgan's points. As to the question of whether our media as we have them today suffer from inadequacies because of deficiencies or because of imbalances, I don't believe these two questions are separable.

I think that, inevitably, all our media either have deficiencies and imbalances in the beginning or have developed them in the course of their use when the cells act upon them. Everything we do in this area is to some extent a compromise.

But I think, as Dr. Morgan himself said, we are moving from an era in which we perhaps had a rather naive idea that a single kind of chemically defined medium would be adequate for all types of cells into an era in which we are specifically looking for substances which govern—to use Dr. Amos' term—or affect the specific function, differentiation, or growth of specific kinds of cells. We are looking forward now to the time when we are going to tailor our medium more specifically than we have done in the past to specific cell types, to specific cell functions.

Dr. Wilde: I should like to stress an empirical point, not covered in Dr. Waymouth's presentation, and that is the role of osmotic pressure in the maintenance of fully differentiated cells.

In the maintenance of fully differentiated heart cells in the absence of proliferation, but maintaining contraction and cross-striation, and so forth, it has been found that appropriate osmolality is around 312 milliosmols. There is a range in here and we don't know why it is 312 milliosmols, but the evidence in our laboratory is that if it drops below 280, the cells do not behave very well, and if it goes above 320, the cells do not behave very well.

Of course, osmolality is simply a gross measure. It is a convenient measure, by instrument, of the total strength of active molecules in any incubation medium. It is yet to be determined whether this is a portion of the sodium-potassium transport mechanisms across the cell, whether there is complexing with the materials that are put out from cells, and so forth. Be that as it may, we found that for the maintenance of differentiated cells, it is wise to keep a very close eye on the osmotic pressure.

Dr. Waymouth: I did, indeed, skip reference to the importance of osmolality in my presentation, but not because I think it is unimportant.

I would remind the audience of another very interesting example of the dependence of cell survival on conditions of osmolality: Dr. Trowell's observations on the extremely low osmolality which is apparently optimal for lymphocytes—an intriguing and I think so far unexplained observation. This particularly stresses the fact that cells of different origins may well have different optimal osmotic environments. The need for this *in vitro* is certainly intriguing. But even more so, I think, is the mechanism by which cells *in vivo* may establish different ion concentrations in their environments. I would like to hear from some of the people who are interested in ion transport.

Dr. Amos: I want to ask Dr. Wilde a question: Does the relative proportion of potassium and sodium affect your possible range?

Dr. Wilde: We have no data on that as yet. This is under study at the moment. This is one of those things that grow up in a laboratory. You become frustrated, you decide you will test osmolality, and you begin to work with it. The osmometer turns out to be the most heavily used instrument in our laboratory now.

Dr. Littlefield: Dr. Waymouth mentioned some work of ours done 2 or 3 years ago and suggested that the DNA polymerase was shifting into the nucleus at the time of DNA synthesis. I have less enthusiasm for this interpretation than I had 2 or 3 years ago.

What we were working with then was synchronous cultures of L cells. We took aliquots of cells before and after DNA synthesis, sonicated and separated them into a soluble and a particulate fraction, and studied the DNA polymerase in these two fractions. We found that there was a statistically significant increase in the particulate polymerase and a decrease in the soluble polymerase at the time of DNA synthesis. After we published this information, we pursued our study a good deal further, trying to separate nuclei morphologically. We never were able to

complete these experiments to get an increase in the nuclear DNA polymerase as we had in the sonicated preparations. So I began to believe that perhaps the answer was in sonicating nuclei which then had more DNA during the S period. We were opening up DNA for nonspecific binding of polymerase. So I am less enthusiastic about this concept. I think, however, the particulate DNA polymerase is a much more important subject for study than in the past.

We recently, along with other people, have emphasized the fact that protein synthesis is coupled to DNA synthesis in mammalian cells. I really think the next step would be for somebody to develop a cell-free system in which DNA synthesis and protein synthesis were coupled.

Dr. Ham: I shall make two comments based on experience with clonal growth in completely synthetic media. First, I would like to underscore the point made by Dr. Waymouth that clonal growth is a very critical test for adequacy of a medium.

Those of you who have followed nutrition are aware, I am sure, that it is very difficult to demonstrate a requirement for biotin. This has been accomplished by Dr. Sanford's group¹, but only through the use of careful starvation over a number of transplant generations.

Figure 1 shows clonal growth in synthetic medium, with and without biotin, of a Chinese hamster line that had previously been grown in a serum-containing medium. Normal colony formation in this system requires cell multiplication well in excess of 1000-fold. As the photograph shows, the controls without added biotin quickly became depleted, whereas in the other culture, with only a very small amount of biotin added (3×10^{-10} M), the growth was very good.

My second point has to do with the question on balance of nutrients raised by Dr. Morgan. Many people have found that tryptophan has been a highly toxic amino acid. Yet, others have found that large amounts are tolerated. We have encountered both conditions. In our medium F-10, tryptophan turned out to be highly toxic, whereas in the more recent medium F-12 large amounts of it were tolerated.

We decided to investigate this further and found that, when the other amino acids in medium F-12 were reduced, tryptophan again became toxic. Specifically, six amino acids were found to be involved in reversing tryptophan toxicity: leucine, isoleucine, valine, phenylalanine, methionine, and tyrosine. By changing the amount of tryptophan in the medium, we could increase the amounts of each of these six amino acids needed for growth. We then decided to ask the question the other way around and study in detail what tryptophan does to the requirement for each of the other amino acids.

I will present only data for leucine here. In normal media, including unaltered F-12, leucine is required for growth with a fairly broad optimum and is toxic only at rather high concentrations. However, in the reduced amino acid medium, where tryptophan is particularly toxic, we found that leucine was also very toxic, with inhibition of growth at 10^{-4} M leucine. On the other hand, when we added an abnormally large amount of tryptophan to the complete medium, which normally gives a broad optimum response, we found that a much higher concentration of leucine was required for adequate growth. There is only a small range of leucine concentration in which growth can be obtained in all three of these media, and for two of them it is suboptimal. Therefore, there is no optimum leucine concentration. The optimum concentration of leucine for growth is highly dependent on the other amino acids in the medium. We have similar results for some of the other amino acids in the group.

Dr. Amos: I would like to comment on this to the extent of some work that Dr. Eaton in our Department has been doing. He has shown that there is some really

¹ Biotin, B₁₂, and other vitamin requirements of a strain of mammalian cells grown in chemically defined medium. Sanford, K. K., Dupree, L. T., and Covalensky, A. B.: *Exp Cell Res* 31: 345-375, 1963.

growing question about whether, or to what extent, animal cells can concentrate amino acids. That is, if they do have permeases, the permeases seem to have very high Michaelis constants. They require high concentrations for saturation of the permease. What is shown is that as he increases the level of amino acid he keeps on increasing the internal pool to extremely high levels. He can also modify the pool of any one amino acid by the other amino acids he puts in it. I don't know whether you have looked at the pools at all in your cells.

Dr. Ham: No.

Dr. Amos: But it is clear that when you have amino acid present, its concentration inside the cells is going to be a reflection, not only of its absolute concentration, but also of other interfering and facilitating amino acids.

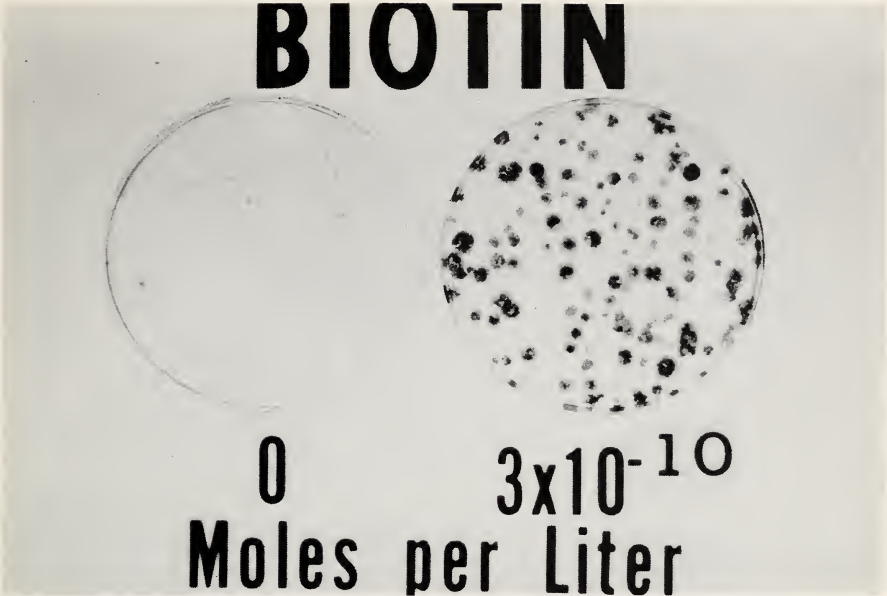


FIGURE 1.—Clonal growth of strain CHL-1 Chinese hamster cells in synthetic medium F-12, with and without biotin.



Evidence for a "Governor" on Protein Synthesis in Animal Cells: Positive Signals in Serum^{1, 2}

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SUMMARY

We propose that most cells in the body are restrained with respect both to the synthesis of proteins and to multiplication. In fact the selection pressure in the evolution of higher animals has favored the development of cells so restrained. Cell division is virtually restricted to what is required to maintain fixed tissue and organ limits, and the cells probably function only on receipt of specific signals from other cells. This means that the performance of almost any cell is not self-generated but a response to requirements at that moment of the whole organism. Our work has dealt with two principal aspects of cell control. First we have shown that normal cells require serum to synthesize proteins. The role of serum is to provide "activators" that

"turn on" machinery already poised in the cell. The machinery is kept under wraps by a "governor." Steps can be taken to release cells from control by the "governor" which probably does not exist in cancer and free-living animal cells. Our second principal interest concerns efforts to keep specialized cells (liver, kidney, intestine) in a normal functional state outside the body. We believe that this can be done only with the "right" pattern of signals for each cell type. Present methods do not provide for such patterns of signals and new procedures must be sought. The implications of these proposals reach as far afield as the problem of tissue and organ regeneration, aging, etc.—*Nat Cancer Inst Monogr* 26: 23–48, 1967.

I SHALL present some experimental results leading to speculation about regulation of protein synthesis in cells of higher animals. I will distinguish between cells in primary culture and those mutants selected for their ability to multiply relatively freely in continuous culture. I have not chosen this particular discussion for its own sake entirely. In fact, I con-

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

² This investigation was supported by Public Health Service research grant AI-09957 from the National Institute of Allergy and Infectious Diseases, and by grant E-348 from the American Cancer Society, Inc.

³ Holder of a career development award from the Institute of Allergy and Infectious Diseases.

clude with some implications of the evidence for future efforts to maintain differentiated cells in culture.

The subject area I was asked to explore is "*in vitro* acclimation and the influence of nutrients." I propose that differentiated cells from higher vertebrates must be fed not only certain amino acids, vitamins, and hormones but also an array of as yet unrecognized molecules, most of which serve as positive signals to maintain the differentiated phenotype. There are surely negative signals as well, perhaps less important numerically, that we cannot yet demonstrate convincingly.

EVOLUTION OF DIFFERENTIATED CELLS

I shall begin my case with some generalizations that are on the whole valid for the regulation of protein synthesis in bacteria. Consider the non-motile bacterium that after millions of years of mutation and selection populates a given plot of soil. It represents that complex of genetic modification best suited to utilize as carbon and energy source the organic materials that are present or are likely from statistical considerations to fall or be washed into its territory. For the utilization of any predominant stable or cyclicly available carbon and energy sources, the current cell represents the most efficient mutant—the fastest growing in effect—that has evolved. In other terms it is the cell which can put that compound to best use, which implies that it also enjoys the least wasteful internal regulation of protein synthesis under the particular environmental conditions.

In *Escherichia coli* the presence of glucose not only assures its efficient use but results in the virtual cessation of the synthesis of certain enzymes, for example, β -galactosidase, glycerolkinase involved in energy derivation from lactose and glycerol, respectively (1, 2). The cell will not utilize lactose or glycerol even if they are abundant in the environment. From a certain point of view the bacterium is master of its internal function with the almost singular objective of multiplication and survival as an isolated entity. Selection has rewarded the tightest effective internal controls that permit maximal adaptability to a changing supply of nutrients.

This image is oversimplified to emphasize the contrasting selective pressures that have operated to produce an animal dependent on populations of differentiated cells. Cell division in higher animals is virtually restricted to what is required to maintain fixed tissue and organ limits. The cell is called upon to exercise its specialized function, often on command from some other organ or tissue. The individual cell is selected not for its ability to multiply but for its sensitivity to signals that define its role in the changing state of the whole organism.

I propose that the selection has favored the plasma membrane as an important site of specific interaction for certain signals. At least four bodies of evidence can be cited to support this proposal. First is the well-established fact that primary cultures can synthesize proteins, and occasionally divide in culture when attached to a surface of glass or plastic, but will do neither if kept in suspension (3). The same cell returned to a surface will recover its synthetic functions in the same medium used for the suspension culture. Undeniably, conformation of the plasma membrane is likely to be a primary feature of this reversible change in responsiveness.

Second are the implications of the relatively large molecular size of some known signals:

1. Adrenocorticotrophic hormone	4,500 (4)
2. Growth hormone (bovine)	45,000 (5)
3. Thyroid-stimulating hormone	26,000-30,000 (5)
4. Insulin	5,800 (6) (polymers of 12,000-48,000)
5. Leuteinizing hormone	26,000-30,000 (5)
6. Prolactin	25,000 (5)

These molecules, though taken up by cells, are probably taken up slowly. It appears unlikely that the response of the cell could be entrusted to ensue from the molecules that have penetrated the cytoplasm.

A third body of evidence concerns the effect of antibodies to cell surface components in the absence of complement (7, 8). Cell growth can be sharply inhibited in the absence of cell lysis by such antibodies. The counterargument could be made that the antibody, by interaction with the membrane, interferes sterically with important functional molecules involved in transport of essential nutrients or prevents the excretion of toxic materials by the cell. Alternatively, the attachment of antibody so modifies the configuration of some receptor sites that they are unable to interact with the specific signal molecule.

Fourth is the evidence of Mitchell and Abbot (9) that antigen is localized at the membrane of potential antibody-forming cells. Though the argument rests on the interpretation of localization of labeled antigen at the cell surface, it is strengthened by the failure of several groups (10-12) to demonstrate antigen molecules in cells actively producing antibody.

To terminate the speculative portion of this paper I propose that many of the positive signals have short half-lives, indeed must have, to terminate their effect soon after the sending tissue has ceased sending. I cite the example of insulin which appears to promote growth and protein synthesis in a variety of cell types in culture (13). Its estimated half-life in the blood is about 20 minutes (14). Indeed, it is interesting to note the reported half-lives in the circulating blood of several of what could be termed "positive signals."

Hormone	Half-life (animal)
1. Growth hormone	20-28 minutes (15, 16)
2. ACTH	10-15 minutes (17)
3. Parathyroid hormone	25 minutes (18)

Some of these substances exist in both a bound and free state in serum and the effective half-life may not be so readily ascertained. Other hormones, such as leuteinizing hormone, prolactin, follicle-stimulating hormone, thyroid-stimulating hormone, and erythropoietin, are known to be labile at 37 C, but half-life determinations are not available.

We envisage the animal cell *in situ* as having major internal restrictions on its protein synthesis that are relieved by general and specific positive signals, many of which are found in serum and interact with the cell membrane. The differentiated cell depends on such positive signals, a number of which are relatively labile, to maintain the phenotype. Absence of the full complement of signals from processed serum may explain our repeated failure to maintain differentiated cells *in vitro*. Lines of cells selected for continuous cultivation *in vitro* have lost the requirement for serum signals to varying degrees.

EXPERIMENTAL RESULTS

Experimental evidence obtained in my laboratory over the past 3 years (with the collaboration of Dr. Ruy Soeiro, Dr. Liane Reif, Dr. K. Ishikawa, postdoctoral fellows; Mr. Arthur Schwartz, a graduate student; Mrs. Ruy Soeiro, Mr. William Christopher, Mr. Anthony Morrissey, Mr. Jean Tersac, Mrs. Mary Moore, and Miss Katherine Kearns, research assistants) leads us to conclude that a significant fraction of protein synthesis is inhibited at the polysome level and that serum contains a variety of positive signals, some of which activate polysomes. Others can be shown to initiate synthesis of specific mRNA, and Dr. Reif will present evidence bearing on such a role for hydrocortisone.

Since my paper was submitted, two reports on cortisone induction of enzyme synthesis in cells in culture have been published. Dr. Moscona (19) showed that hydrocortisone induces a synthesis of the enzyme, glutamotransferase, in chick retinal cells. Dr. Reif has been working with this same system. Dr. Gordon Tomkin's group (20) recently demonstrated a role for steroids in the induction of the enzyme tryosine α -ketoglutarate transaminase in a hepatoma cell line.

The observation that introduced us to this area was a confirmation of the oft-reported requirement of serum for the growth of primary cultures. There is, of course, the important exception of Dr. Rappaport's growth of monkey kidney cells on specially treated glass (21). We suspect that

her success is explainable by the large inoculum of cells she used. Even with large inoculum and treated glass very few, if any, dispersed cells can be made to multiply in primary culture without serum proteins. And as you well know success is limited, to say the least, despite the complicated additives to which tissue culturists have become addicted. Dr. Foley (22), using modified Eagle's medium and adult or fetal serum, has had consistent success with the cover-glass method (23, 24). I shall not linger on this aspect of the problem and apologize to those responsible for a good deal of valuable work that I lack time to mention.

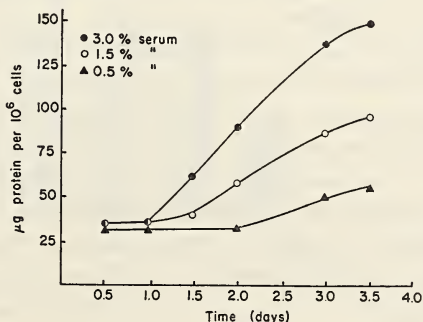
Whereas most of the work previously reported concerned cell multiplication, we confined ourselves to a study of protein synthesis in essentially nondividing cells. Most of our work was done with chick embryo fibroblasts, though human amnion and mouse-embryo primary cells as well as L and HeLa cells were sometimes used.

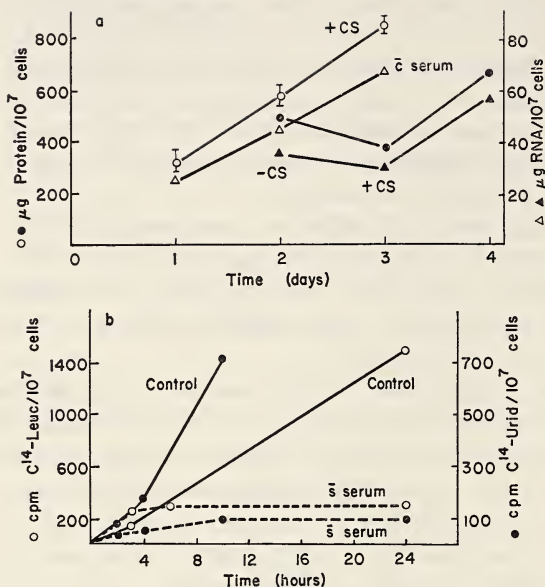
The chick cells in Eagle's basal medium require serum for net protein synthesis, and at serum concentrations up to 5%, the rate of synthesis established is a function of the serum level (text-fig. 1) (25). At a given serum level synthesis goes on at a constant rate for several days. If serum is removed at any point, net protein synthesis ceases abruptly and the incorporation of protein precursors into polypeptides is reduced by 60-80% (text-fig. 2) (26). RNA synthesis suffers a similar fate, though the pulsed incorporation of ^{14}C precursors (uridine- ^{14}C or adenine- ^{14}C) into polynucleotides is often maintained at 60-70% of normal. There is a high rate of RNA turnover of a limited portion of the cellular RNA. Net synthesis of both protein and RNA resumes within an hour or so of the return of serum to the medium. This capacity survives several days of serum deprivation.

Dr. Soeiro showed with actinomycin D that the capacity to renew protein synthesis after deprivation did not require RNA synthesis during deprivation periods up to 8 hours (text-figs. 3 and 4). That is, the addition of actinomycin D at the time of the withdrawal of serum did not adversely

TEXT-FIGURE 1.—Effect of serum concentration on protein synthesis. Cells obtained from 10-day embryo by trypsinization were distributed to a series of bottles. Two bottles were harvested after careful washing at each point, and proteins determined by the Lowry procedure. Cells were cultured in Eagle's basal medium, and calf serum was added as indicated.

Effect of Serum Concentration on Protein Synthesis





TEXT-FIGURE 2.—Effect of serum removal on protein and RNA synthesis. a) Cells cultured in Eagle's basal medium (BEM) with 3% calf serum for 48 hours were washed twice with balanced salt solution (BSS). To half the bottles fresh BEM with 3% serum was added and to the rest BEM without serum. (C.S. = 3% calf serum.) Cells were harvested and proteins estimated by the Lowry procedure. Each point represents the average of 2 bottles of approximately 10^7 cells per bottle. b) ^{14}C -leucine ($0.25 \mu\text{c}/\text{bottle}$) or ^{14}C -uridine ($0.25 \mu\text{c}/\text{bottle}$) was incorporated into cold TCA-insoluble fraction. Labeled precursors were added at time of medium replacement. Estimate of cumulative incorporation was made by harvesting 2 bottles for each point indicated.

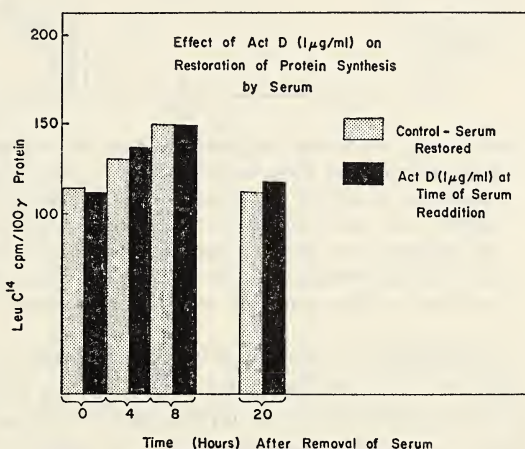
affect the ability of cells to reinitiate protein synthesis 4 or 8 hours later compared to controls. The toxicity of actinomycin precluded its use for longer periods.

Our tentative conclusion was that mRNA was not destroyed during the period without serum and that the mRNA remained in a polysomal structure or could reform polysomes after the return of serum.

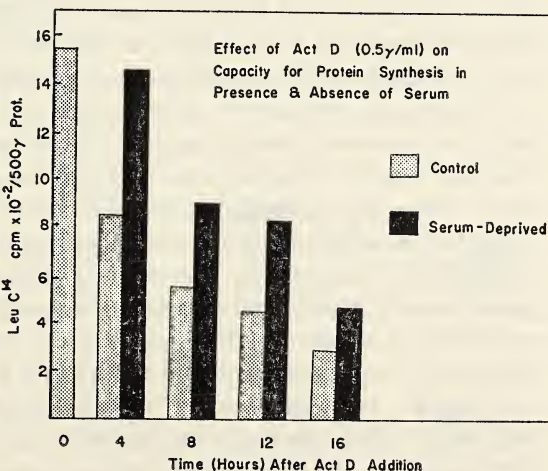
Dr. Soeiro then compared by sucrose gradient centrifugation the ribosome pattern of cells with and without serum and found it essentially identical (text-fig. 5) (26). There were in both cases a large polysome component and a significant fraction of monosomes. At the same time he determined the functionality of the ribosomes from both types of cells and found those prepared from serum-starved cells only 30–40% as active as their serum-fed counterparts (text-fig. 6). Indeed the ribosomes from the starved cells were virtually unresponsive to stimulation of ^{14}C -phenylalanine incorporation by polyuridylic acid, while the more active ribosomes were markedly stimulated. These findings have been confirmed and extended by Mr. Arthur Schwartz in a direction which I lack time to discuss. Finally, Dr. Soeiro showed that when the fractions of ribosomal aggregates separated by sucrose gradient centrifugation were used separately for *in vitro* incorporation of amino acids into polypeptides, the

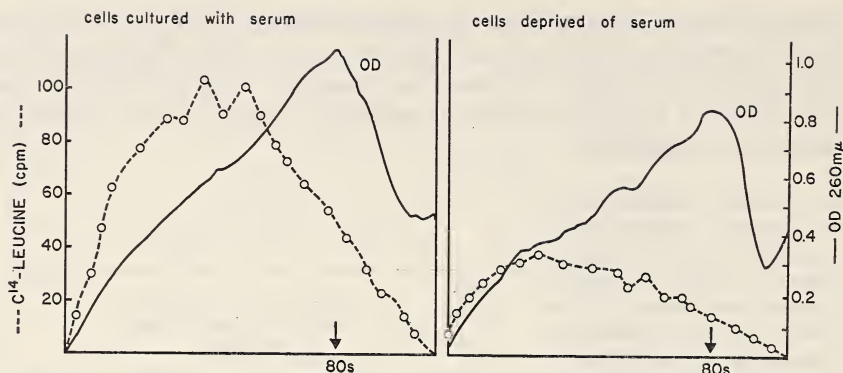
most active elements came from the polysome region of the gradient (text-fig. 5). Again elements from that region were less active if prepared from cells deprived of serum. We conclude from this body of evidence that when serum is removed the polysomes remain aggregated but synthesize proteins at a reduced rate.

TEXT-FIGURE 3.—Effect of actinomycin D on restoration of protein synthesis by serum. After 2 days in culture, serum was removed (time zero) from the medium as shown in text-figure 2. At various times thereafter, serum was restored to the medium with or without actinomycin D ($1.0 \mu\text{g/ml}$) and with ^{14}C -leucine. After 4 hours the cells were harvested and the ^{14}C -leucine incorporated into TCA-insoluble material was measured. ^{14}C -uridine incorporation into actinomycin D-treated cells was less than 8% of control (first 4 hours after actinomycin D). Protein was estimated by the Lowry procedure.



TEXT-FIGURE 4. — Effect of serum deprivation on persistence of capacity for protein synthesis. Actinomycin D ($0.5 \mu\text{g/ml}$) was added at zero time to cultures freshly transferred to medium with or without serum. After such preincubation for varying intervals, serum was added to the serumless cultures and the amount of ^{14}C -leucine incorporated into TCA-insoluble material in the subsequent 4 hours was determined in both cultures. ^{14}C -uridine incorporation into actinomycin D-treated cultures was less than 8% of control (first 4 hours after actinomycin D). Protein was estimated by the Lowry procedure.



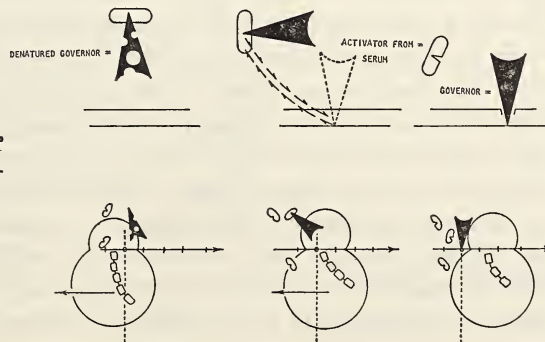
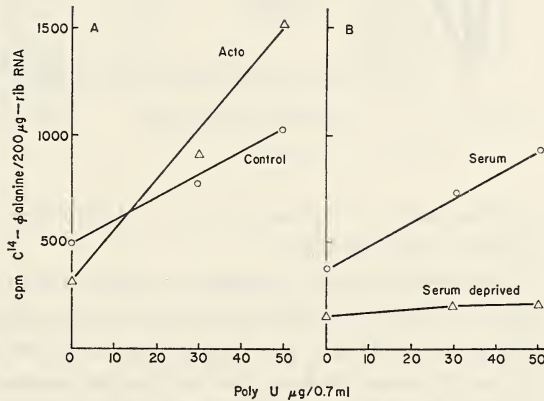


TEXT-FIGURE 5.—Amino acid incorporation into polysomes of serum-starved and normal cells. Microsomes from cells cultured in 3% serum and from cells of the same population deprived of serum for 24 hours were treated with Na deoxycholate (0.5%) and subjected to sucrose gradient fractionation at 0 C. Fractions from the gradient were used as ribosomes for *in vitro* endogenous amino acid incorporation with a normal supernatant fraction from whole chick embryos. Conditions for incorporation were the same as those given in text-figure 6.

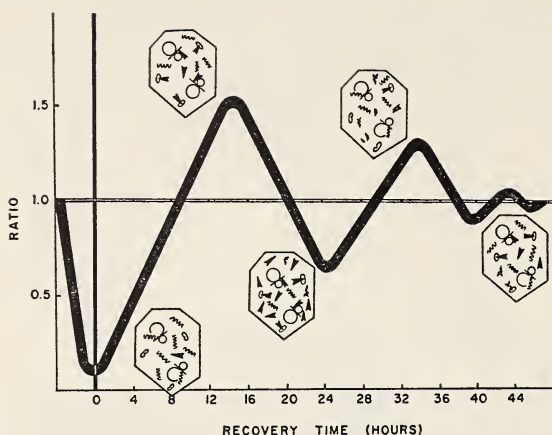
In seeking to explain the role of serum in a reactivation that did not require RNA synthesis, we returned to a repeated finding of accelerated protein synthesis in the early hours following restoration of serum (text-fig. 3). Again the "overshoot," as we call it, was not dependent upon RNA synthesis. We surmised then that we were dealing with a "governor,"—perhaps protein in nature—on the polysome, and that serum contained an "activator," which functioned directly or indirectly to neutralize the "governor" (text-fig. 7). The suppression of protein synthesis for several hours would result in a reduced concentration of "governor" (provided it is a protein of relatively short half-life) permitting a higher rate of protein synthesis when serum was restored. On the other hand, we could have been dealing with a special effect of serum.

If indeed the "governor" were a protein of a half-life of say 2 hours, the interruption of protein synthesis for 4 hours or more, without other resultant injury to the cell, would effect a lowered concentration of "governor." Recovery under the influence of normal "activator" concentrations would be characterized by an "overshoot" when the inhibitor was removed. Mr. William Christopher, who actively pursued these experiments, suggested we might very well detect a second period of inhibition, due to the accumulation of abnormally high levels of "governor," a second "overshoot," etc.; in other terms, an undulating course of return to normal (text-fig. 8), all accounted for by the changing ratio of "governor" to "activator." Since the activator concentration is a function of serum concentration, it can be considered to remain constant throughout the recovery period. Hence, the fluctuations of rate of protein synthesis essentially re-

TEXT-FIGURE 6.—Amino acid incorporating activity of actinomycin D-treated and serum-starved microsomes. Microsomes were prepared by gently disrupting cells in a Potter-Elvehjem homogenizer; after a clearing spin, the microsomes were collected as a $100,000 \times g$ pellet. Microsomes from cells exposed to actinomycin D ($0.1 \mu\text{g}/\text{ml} \times 16$ hours) or cells deprived of serum (24 hours) were compared with microsomes from untreated cells, in the presence of normal whole-chick-embryo supernatant. Incubation for 40 minutes at 37°C . Total volume 0.7 ml ; mercaptoethanol $.006 \text{ M}$; ATP $0.9 \times 10^{-3} \text{ M}$; creatin phosphokinase $28 \mu\text{g}$; creatin phosphate 1.0 mg ; GTP 10^{-4} M ; KCl $7.6 \times 10^{-2} \text{ M}$; MgAc $5 \times 10^{-3} \text{ M}$; ^{14}C -phenylalanine $0.25 \mu\text{c}$; 19 amino acids ($0.025 \mu\text{M}$ in each); approximately 1.5 mg of S-100 protein and $100\text{--}200 \mu\text{g}$ of microsomal RNA; poly U (Miles Chemical Co., Elkhart, Ind.) as indicated.



TEXT-FIGURE 7.—Scheme of "governor" inhibiting protein synthesis.



TEXT-FIGURE 8.—Projected course of protein synthesis in recovery after prolonged inhibition of protein synthesis.

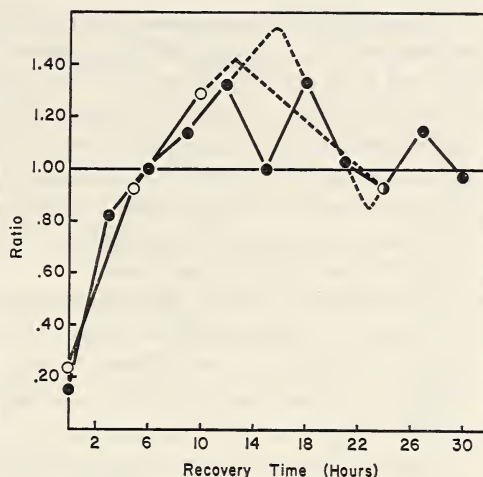
flect the level of "governor"; *i.e.*, the rate is inversely proportional to governor concentration.

To test this model, protein synthesis was inhibited by cycloheximide and by *p*-fluorophenylalanine, as well as by removal of serum. The rate of subsequent synthesis was determined at intervals during recovery for as long as 30 hours. Indeed, the undulating course was observed as predicted (text-fig. 9). Although experiments with *p*-fluorophenylalanine and serum withdrawal were not as extensive, the pattern of recovery confirmed that demonstrated following cycloheximide inhibition.

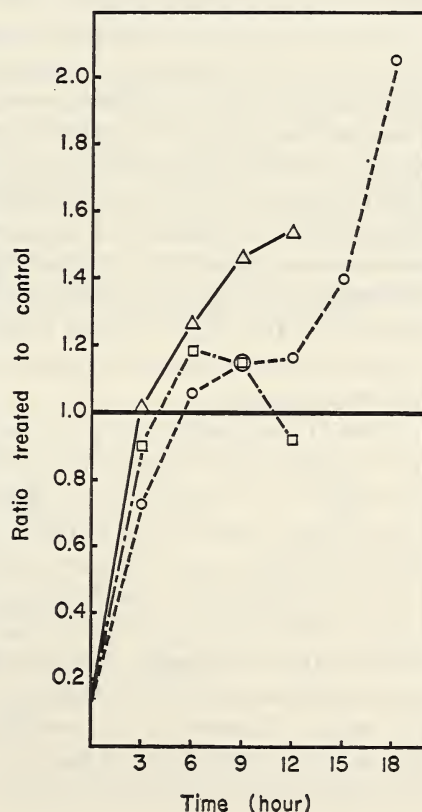
Cycloheximide (27) leaves polysomes intact, and results obtained with actinomycin D (text-fig. 10) indicate that RNA synthesis is not required either during the period of inhibition or during recovery to demonstrate the "overshoot." In fact, the presence of actinomycin D appears to magnify the effect and to delay, if not prevent, the return to normal. One might be tempted to propose that the mRNA that codes for "governor" is, like the "governor" protein itself, of short half-life. The synthesis of new "governor" would thus be relatively more seriously inhibited by interference with RNA synthesis than would general protein synthesis.

One further prediction based upon the assumption of a protein "governor" has been tested. If an amino-acid analogue were incorporated in place of the natural compound during recovery, and if the altered "governor" protein proved less effective than the normal protein, a greater and prolonged overshoot should result. This is, in fact, what takes place when DL-*p*-fluorophenylalanine (200 $\mu\text{g}/\text{ml}$) is supplied in place of phenylalanine during recovery from cycloheximide inhibition (text-fig. 10). Inhibition caused by the continued presence of fluorophenylalanine made events subsequent to 15 hours difficult to interpret.

TEXT-FIGURE 9.—Recovery of protein synthesis after inhibition by cycloheximide. Cells were cultivated 72 hours in Eagle's medium + 3% calf serum. Cycloheximide (0.5 $\mu\text{g}/\text{ml}$) was used to inhibit protein synthesis for 4 or 12 hours. The cells were washed with balanced salt solution and warm fresh medium without cycloheximide. ^{14}C -phenylalanine was added for 3-hour incorporations. The ratio is that of ^{14}C -phenylalanine incorporation per 100 μg of protein in experimental cells compared to that of untreated controls also washed and fed fresh medium.



TEXT-FIGURE 10.—Effect of actinomycin D and of *p*-fluorophenylalanine on "overshoot" during recovery from cycloheximide inhibition. Experimental procedure as described for text-figure 9. Δ Actinomycin D (0.5 $\mu\text{g}/\text{ml}$) was added both to cycloheximide-inhibited and control cells from the start. Protein synthesis was inhibited for 4 hours with 5 $\mu\text{g}/\text{ml}$ cycloheximide. Cells were washed and actinomycin was returned to all cells. $\circ$ DL *p*-fluorophenylalanine (200 $\mu\text{g}/\text{ml}$) was substituted for phenylalanine in the recovery medium of cells exposed to 5 $\mu\text{g}/\text{ml}$ cycloheximide for 4 hours as well as to cells not inhibited by cycloheximide. $\square$ Cycloheximide for 4 hours followed by normal medium in recovery. The ratio represents ^{14}C -isoleucine incorporation during 3-hour periods.



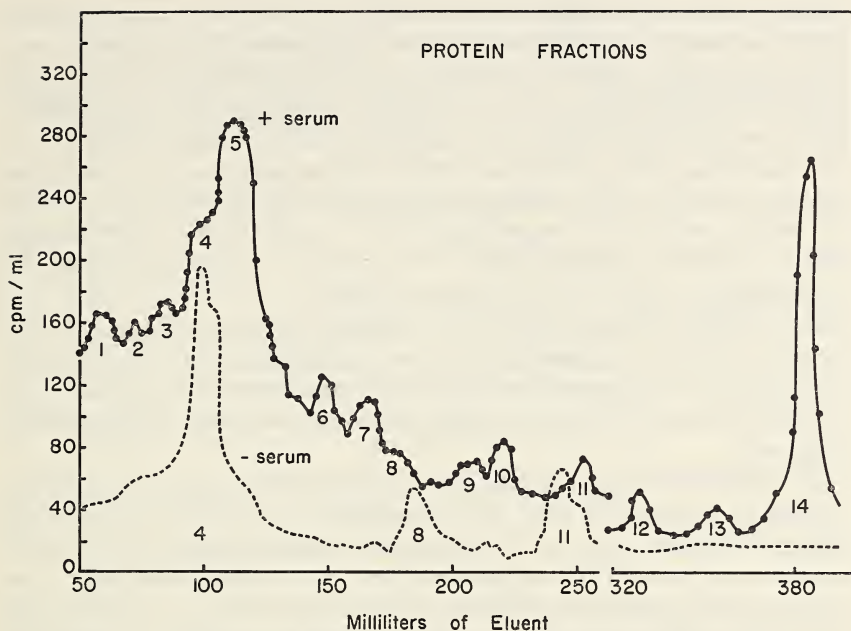
The scheme we have come to formulate is one of cells *in situ* internally constrained by a "governor" or a series of "governors" which can be neutralized by a general or specific "activator(s)." This means that the cell, even with genes activated to make mRNA and possessing polysomes, is still dependent upon a constant stream of signals (activators) either in the serum or supplied directly by neighboring cells to maintain its phenotype. Moreover, we are already aware of the necessity for signals to induce gene function, some general (cortisol) (28) and some specific (29). At least some of these also are in serum.

Where has this led us experimentally? We have elected to demonstrate that serum contains positive signals that turn on the synthesis of specific proteins. Ideally one would search for several identifiable proteins, preferably enzymes, each of whose synthesis by the cell could be shown to require a specific serum component.

In such a venture the gamble involves choosing the right enzyme and the corresponding signal. We approached the problem first by scanning the cell protein in a crude way, hoping to obtain several proteins or several classes of proteins to observe. Amino-acid labeled chick-cell proteins were eluted from DEAE-cellulose columns with a series of gradients employing changes both in pH and in ionic strength (30). Reproducible patterns of some 30 or 40 distinct peaks were obtained, of which 14 are shown in text-figure 11. If the cells first grown in serum were provided with serum-free medium during the period of ^{14}C -amino-acid incorporation, the reduced synthesis of proteins did not suppress all classes of proteins made. Some fractions, in this portion of the profile 4, 8, and 11, were actively synthesized in the absence of serum, whereas many more were made when serum was supplied. This clear and reproducible finding can be considered presumptive evidence for positive signals in serum, and extension and refinement of this plan of attack should permit the assessment of proteins whose synthesis is influenced by such molecules as insulin, growth hormone, hydrocortisone, etc.

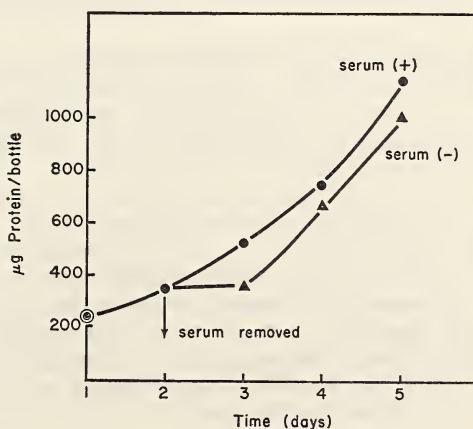
I want to emphasize that our interpretation for the moment is confined to the proposal that these data are evidence for positive signals in serum that enhance the synthesis of a variety of proteins. We are unable as yet to say at what level of the DNA-to-protein sequence the signals act (31). That information may not prove too difficult to obtain.

Dr. Ishikawa found that L cells growing as monolayers provided with serum continued to multiply when serum was removed (text-fig. 12). Preliminary experiments indicate that these cells, when provided with serum, make several proteins not needed for growth and multiplication and not made in the absence of serum. Thus it appears that the L cell, which has no serum requirement, after years of continuous culture *in vitro*, still responds to positive signals in serum.



TEXT-FIGURE 11.—Protein profile-gradient phase 1: 310,000 counts per minute (cpm) of ^{14}C -labeled protein (20 mg) from cells incubated 48 hours with serum and 80,000 cpm (16 mg) protein from cells incubated 48 hours without serum were applied to separate DEAE cellulose columns in 0.005 M sodium phosphate, pH 7.2. Both lots of cells came from the same starting population and all were preincubated with serum before exposure to ^{14}C -isoleucine. Gradient phase 1 (400 ml): 0.005 M sodium phosphate, pH 7.2, to 0.02 M sodium phosphate, pH 6.0; gradient phase 2 (400 ml): 0.02 M sodium phosphate, pH 6.0, to 0.05 M sodium phosphate, pH 6.0; gradient phase 3 (400 ml): 0.05 M sodium phosphate, pH 6.0 to same plus 0.02 M NaCl. Flow rate was maintained throughout at approximately 60 ml/hour.

TEXT-FIGURE 12.—Effect of serum withdrawal on protein synthesis in L cells. Monolayers of L cells in Eagle's minimal medium (+10% horse serum) were deprived of serum after 2 days in culture. Two bottles of cells were harvested daily and protein content was determined by the Lowry procedure.



I shall deal only briefly with the implications of the role of positive signals in serum, though I believe they may prove of considerable consequence. To begin with, if the maintenance of the phenotype of a differentiated cell *in situ* should require a constant stream of the right positive signals, we have little chance of cultivating differentiated cells with our current media. Many of the hormones cited earlier have short half-lives and they, as well as other unknown labile molecules, are unlikely to survive our crude methods for preparing serum. Even with the right starting material, constant perfusion (31) will probably be required to keep the proper balance of signals impinging on the cells.

An alternative is to provide mixed feeder layers of some complexity, since the suspicion is growing that almost all tissues of the animal body participate in sending positive signals. A feeder layer of a single cell type is unlikely to provide any significant proportion of what is required by a given cell.

We have been too much influenced to think of animal cells as larger editions of *Escherichia coli*, able to maintain their identity in minimal medium. We have tried to encourage the cells to maintain their special functions and to multiply at the same time in a medium essentially of an energy source, precursors to macromolecules and vitamins. Indeed a great deal of the effort in recent years has been aimed at providing a completely defined medium, free of protein. Differentiated cells can be counted on to "dedifferentiate" in culture, and we have come to attribute this to sloth or obstinacy on their part. It is equally possible that the differentiated cells are signal-dependent and that we have failed to provide the proper complex of signals in culture. What signals can be identified and their role in maintaining the differentiated phenotype may prove an interesting area for investigation in the years immediately ahead.

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DISCUSSION

Dr. Morgan: For the reason given in my discussion of Dr. Waymouth's paper, I am particularly interested in Dr. Amos' description of his use of freshly explanted chick embryo fibroblasts. For many years we have been working on the nutrition of chick embryo heart fibroblasts cultivated in chemically defined medium. In 1955 we found that the addition of very small amounts of many natural materials to such a culture system caused a tremendous increase in the survival of our cell cultures. When we studied protein synthesis in this system, we found that a net loss of protein occurred when our chick embryo explants were maintained in synthetic medium 199. This loss of protein could be prevented, as we reported in 1958, by the addition of many materials of biological origin to the chemically defined medium. We found that the loss of protein could be converted into a net gain of protein by a great variety of biological substances. One of these was serum. The interesting observation here was that while serum was certainly very effective in the protein system, it was much less effective than compounds of other types.

For example, 5% serum appeared necessary, but as little as a tenth or sometimes a hundredth or a thousandth percent of other materials, such as yeast extract, chick embryo extract, or liver guanine concentrate, would produce much the same effect.

This introduces another question. Assuming Dr. Amos' concepts are valid, we should remember that serum may not necessarily be the only or the best source of such governors and activators. It is possible that the search for other materials would yield interesting results and modify some of the hypotheses presented.

I should also mention that the extensive publications of Kutsky and Harris on the purification of the unidentified growth factors from chick embryo extract might well relate to Dr. Amos' finding.

There has been, I think, one common theme in the presentations of both Dr. Waymouth and Dr. Amos. In the period since the last Decennial Review Conference, much information about cellular nutrition in general has been obtained and it certainly has been reviewed very admirably by Dr. Waymouth. With the background of this information, we are now moving away from general nutritional problems into a study of the specific nutrition of cell functions. Such studies have been presented extremely capably by Dr. Amos. Opportunities in this area are almost unlimited.

Dr. Amos: I think the evidence from use of yeast, liver, and chick embryo extracts is rather interesting because these are gently prepared extracts. The paper in *Science* of Dr. Harris is a nice one on the column fractionation of the chick embryo extract. I think these materials are prepared much more gently than serum, and I think the fact that smaller amounts are useful is evidence that we have been destroying important things in serum.

Dr. Wilde: Dr. Amos raised a point which is increasingly important to people dealing in developmental areas. Although developmentalists have thought in terms of "hours"—we refer to the first hour or the second hour, and so forth—I want to make a plea for the development of a "minute," or "subminutes," complex. I am bewildered by new data that we obtained this summer pertaining to embryogenesis

in which there is something very important about the 5th minute after fertilization. I make a plea for the fact that these biological reactions and contacts of molecules and behaviors between cells are really occurring on the basis of subhours, perhaps even milliseconds. We all should begin to orient our thinking toward much smaller time intervals than we have been using. I expect that eventual conversion to shorter time periods of chemical reactions will be very important in the future.

I hoped we might have some open and frank discussion about the roles of the common inhibitors we use. Professor Adrian Högman, who was one of my summer colleagues, says he has what he calls Högman's law. Somebody else has a law, which I presume is concerned with it, that the specificity of the inhibitor is in inverse proportion to the number of experiments you have done with it. I particularly make a plea for this for actinomycin D. There is some evidence that it inhibits respiration in homeotherm embryos. In poikilothermic embryos and fish and amphibia, it does not inhibit respiration. In fish, it has profound effects on protein synthesis that are unexpected because it is all encoded. I think actinomycin D probably does much more than just complex guanine residues. I hope to hear some open discussion about how good is an inhibitor.

I should like to hear a contrast made between Dr. Amos' concept, with which I am thoroughly in accord, of exogenous governors and controls that pass in the extracellular milieu of organized cellular systems, and Dr. Waymouth's description of the historical role of heterologous serum. I think it is important to compare this heterologous serum, which people have just simply plastered into cells ever since tissue culture began, with these very remarkable pinpoints that Dr. Amos is coming up with in dealing gently with serum.

I would like to reemphasize the whole question of cellular services to broaden the concept to include all membrane services in cells. Here the ultrastructural people will lead the way by indicating what commonalities there are between the gross surface membranes and the multiplicity of membrane systems that we find in actively synthesizing cells. Are they one and the same? Are there pores that connect the two? How does membrane surface increase? From what moiety does it come? A worthwhile hypothesis is to consider that a large number of cellular interacting systems are service systems that work at services.

Finally, I would like this group to consider what differences there are between homeothermic cells and poikilothermic cells, or amnion cells and amnion cells among the vertebrates. There are differences. There are great puzzlements. And from time to time, I hope this will come up in the discussion.

Dr. Amos: Concerning Dr. Wilde's comment on actinomycin D, I am sure anyone working with actinomycin D is quite aware that it is doing other things. We have also prepared for publication information on certain toxic effects in the very cells we work with. We would be reluctant to interpret negative findings of actinomycin D as really meaning that RNA had to be synthesized. Since in our case actinomycin D permits full expression, the alternative toxic effects, which other people have reported, need not be considered.

In addition, we are certainly trying to do the same things with analogues of the precursors of RNA just to protect ourselves from that sort of thing. I think that is very important.

Dr. Wilde: Just the skepticism about the role of inhibitors is a cogent point.

Dr. Amos: I agree with that.

Dr. McCarty: Dr. Amos' experiments suggest that serum may contain a "host of positive signals to turn on the synthesis of specific proteins." He implied, for example, that even with the "right starting material (*i.e.*, hormones?) a constant perfusion will probably be required to keep the proper balance of signals continuously impinging on the cells." He suggests further that this control is exerted at the polysome level and that "serum contains a variety of positive signals, some of

which activate polysomes" and thereby exert their control at the level of translation of mRNA into protein.

Before we discuss details of these provocative questions, it might be helpful to review some present concepts of the mechanisms of replication, transcription, transfer, and translation of information at the molecular level. In addition, some pertinent experiments from our laboratory will be mentioned.

In bacteria, as well as higher organisms, there is overwhelming evidence for our concept that genetic information within the DNA molecule is transcribed into an mRNA which is later translated into proteins at the mRNA ribosome complex (polysomes) (McCarty, text-fig. 1). Although there is abundant evidence for regulation of protein synthesis at the level of mRNA transcription of DNA, as suggested by Jacob and Monod,¹ this control necessarily involves highly unstable mRNAs with short half-lives. The recent discovery of stable mRNAs, however, both in rat liver² and bacteria,³ necessitates our consideration of additional control mechanisms at the polysome level.

Dr. Amos suggests that such a control exists in chick fibroblasts in tissue culture. Some precedent for this concept is provided by recent experiments of Monroy *et al.*,⁴ which suggest that ribosomes in unfertilized sea urchin eggs are inactive and may only be activated by removal of some of the protein(s) by gentle trypsin treatment.

In our experiment with sea urchin eggs,^{5,6} we noted that protein synthesis is activated within 10 seconds of fertilization, and this activation is not inhibited by actinomycin D.

One question that must now be asked about Dr. Amos' experiment is: Are the same mRNAs present in the inactive chick cell when compared to the active cell? These hybridization experiments have been done, for example, in sea urchin eggs, by Whiteley *et al.*⁷

Dr. Amos implied from observations of the polysome distribution and from the amino acid label, that the polysome profiles do not change in this system. We must be cautious in these interpretations, however, because the two polysome patterns do not appear to be identical, and the resolution is poor for a critical examination (Dr. Amos, text-fig. 5). These patterns must be studied in greater detail.

Dr. Amos' text-figure 2 shows that neither ¹⁴C-leucine nor ¹⁴C-uridine was incorporated into TCA-insoluble material when the cells were grown in the absence of serum. I would like to ask Dr. Amos if he considers this significant. This suggests that neither RNA nor protein synthesis proceeds in cells grown in the absence of serum. Perhaps it would be better in these experiments to include additional controls containing linolenic acid, isoleucine, or polyisoleucine when the chick cells are grown in the absence of serum.

I would like now to present some additional experiments which bear on the subject area of this symposium, "*in vitro* acclimation and the influence of nutrients." These

¹ JACOB, F., and MONOD, J.: Genetic repression, allosteric inhibition, and cellular differentiation. In *Cytodifferentiation and Macromolecular Synthesis* (Locke, M., ed.). New York and London, Academic Press Inc., 1963, pp 30-64.

² REVEL, M., and HIATT, H. H.: The stability of liver mRNA. *Proc Nat Acad Sci USA* 51: 810, 1964.

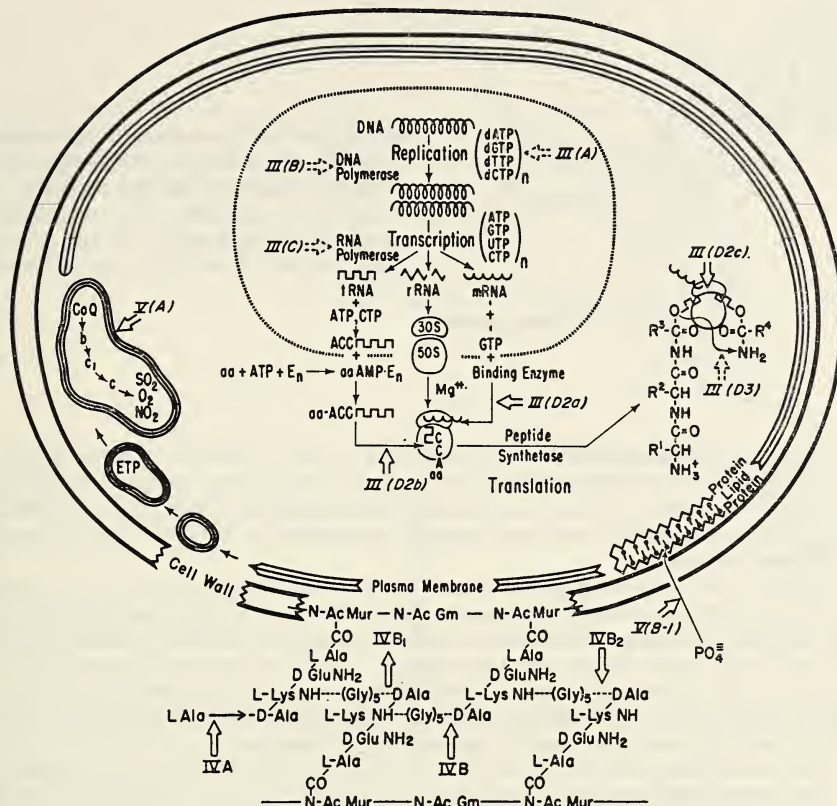
³ YUDKIN, M. D.: Protein synthesis by long-lived messenger ribonucleic acid in bacteria. *Biochem J* 100 (1): 501-506, 1966.

⁴ MONROY, A., MAGGIO, R., and RINALDI, A. M.: Experimentally induced activation of the ribosomes of the unfertilized sea urchin egg. *Proc Nat Acad Sci USA* 54: 107, 1965.

⁵ MCCARTY, K. S., STAFFORD, D., SY, J., and CAMPBELL, C. C.: Control of protein synthesis at the translational level in sea urchin eggs. *J Cell Biol* 31: 75A, 1966.

⁶ STAFFORD, D., SOFER, W. H., and IVERSON, R. M.: Demonstration of polyribosomes after fertilization of the sea urchin egg. *Proc Nat Acad Sci USA* 52: 313, 1964.

⁷ WHITELEY, A. H., MCCARTHY, B. J., and WHITELEY, H. R.: Changing populations of mRNA during sea urchin development. *Proc Nat Acad Sci USA* 55: 519, 1966.



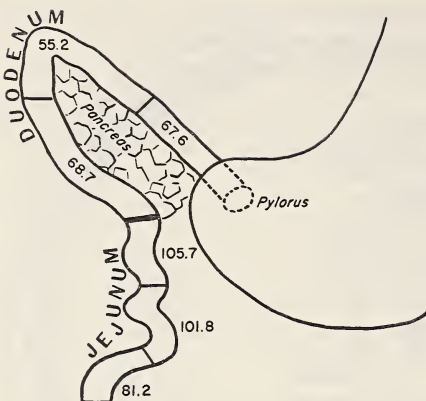
TEXT-FIGURE 1.—Some of the molecular mechanisms of replication of DNA, its transcription into RNA, and the translation of messenger RNA into protein are shown in this diagram. The details of these mechanisms are described by Carter, W., and McCarty, K. S.: *Ann Intern Med* 64: 1087, 1966.

studies were designed to investigate the induction of invertase by hydrocortisone, with chick embryo duodenum in culture. The specific activity of invertase is constant throughout the loop (McCarty, text-fig. 2), providing an excellent control system whereby half the loop may be cultured as control and the other half treated with hydrocortisone (0.5 $\mu\text{g/ml}$). The tissue is placed on Millipore membranes (0.45 μ) and may be cultured for at least 10 days with no evidence of necrosis by light or electron microscopy in the presence or absence of the hormone.⁸

Invertase activity increases with the age of the embryonic tissue examined (McCarty, text-fig. 3), with a maximum activity after 48-hour exposure to the hormone. These studies show that it is possible to place in culture selective tissue (14-day embryos) with little invertase activity and induce the activity of this enzyme with hydrocortisone.⁹ The greatest induction activity is from 14-day tissues that nor-

⁸ DOBBINS, W. O., HIJMAN, J. C., and MCCARTY, K. S.: A light and electron microscopic study of duodenal epithelium of chick embryos cultured in the presence and absence of hydrocortisone. *Gastroenterology*. In press.

⁹ MCCARTY, K. S., HIJMAN, J. C., and WILHELM, J.: Homeostasis in metazoan cell cultures. *Ann NY Acad Sci* 139: 214, 1966.



TEXT-FIGURE 2.—Diagrammatic representation of chick embryo duodenum. The numbers represent specific activities of invertase (expressed as μg glucose formed per mg protein per hr) of various regions of the tissue as indicated.

mally demonstrate little activity *in vivo*. Only a small increment in activity is obtained when 19-day embryo tissue is tested. If the 19-day tissue is first cultured for 2 days in the absence of hydrocortisone, and then an additional 2 days in its presence, it is possible to obtain the same response comparable to that of embryo tissue¹⁰ (McCarty, text-fig. 4).

These experiments suggest that one must be extremely critical of the conditions selected for these particular experiments. In addition, one must be cautious in selecting the embryonic age of the tissue to be tested. A negative modifier (inhibitor) may be present in 19-day tissue, since this enzyme activity can be induced after 2 days in culture in the absence of hormone. Without this hormone control (negative modifier), which may be lost by the culture condition, the resulting levels of invertase activity would become abnormally high in response to the hormone.

Dr. Amos: What you have to say is quite beautiful. This is certainly a refinement on the rather crude things we have been doing. It is a very nice and very interesting paper. But I want to defend myself a bit on one or two points you made. The curves we showed you on the polysomes, about the 25 individual fractions, were cut from these polysomes and tested for their *in vitro* protein-incorporating activity. Although I agree this is a very crude scheme, it still is a double check on the quality of the fractions that have been run through the gradient, then either individually tested for their *in vitro* RNA-incorporation activity with or without messenger.

Dr. McCarty: In your paper you state you used polyuridylic acid.

Dr. Amos: That's right.

Dr. McCarty: Did you also test endogenous activity? The use of polyuridylic acid will stimulate only the monosomes in your polysome preparation.

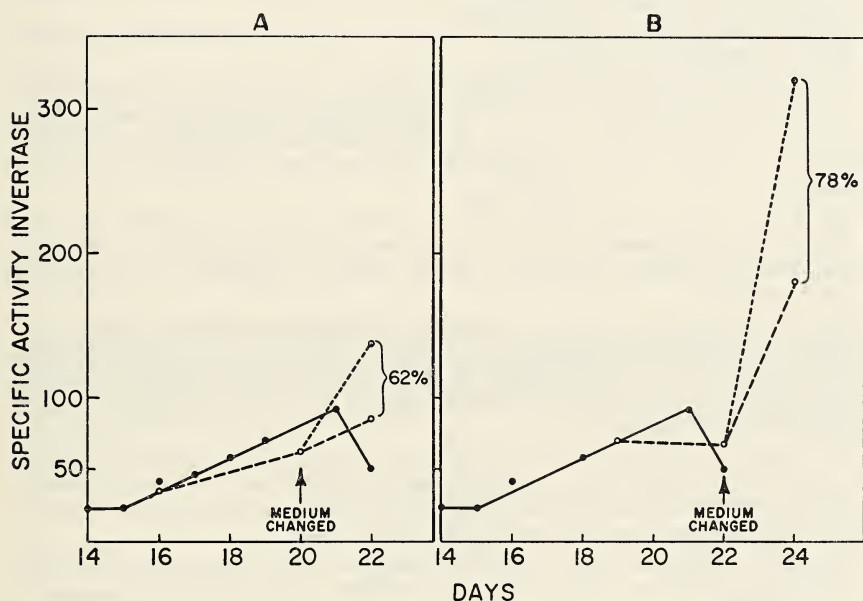
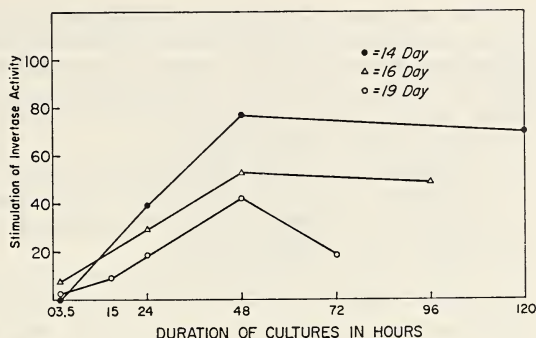
Dr. Amos: The count that I showed is endogenous. I did not refer to the counts, but they are higher on that plot. That was endogenous. There is still some question as to whether polyuridylic acid stimulates only monosomes.

The other point I wanted to make is that I really was guilty, because of time, in not saying that uridine's incorporation is actually for a short time. It doesn't fall away. The net synthesis stops. With RNA, synthesis continued at about 70% of normal. It is just the turnover that is quite rapid.

Dr. Wilde: Dr. McCarty, just exactly what do you mean by a negative inhibitor, a phrase you used—two negatives make a positive? Does this jazz things up, or can we get this one off the record so that we will call an inhibitor an inhibitor?

¹⁰ HILMANS, J. C., and MCCARTY, K. S.: Induction of invertase activity by hydrocortisone in chick embryo duodenum cultures. *Proc Soc Exp Biol Med* 123: 633, 1966.

TEXT-FIGURE 3.—Relative stimulation of invertase activity compared to control culture for 14, 16, and 19-day explants.



TEXT-FIGURE 4.—Specific activities of invertase for explants taken at intervals indicated, cultured first in the absence of hydrocortisone and then in its presence. ●—●: *in vivo* activity. ○—○: tissue in culture medium without hydrocortisone. ●—●: tissue in culture medium with hydrocortisone.

Dr. McCarty: I think this is probably a misnomer. But, if you would consider that everything is already maximally inhibited—for example, all of the DNA in the cell is maximally inhibited—then only portions are being uninhibited. Thus you could say that there is an uninhibitory something that is removing the histone from the DNA so that the DNA can now be read.

Dr. Wilde: Is it a stimulator?

Dr. McCarty: If you want to call it that, it is a stimulator. A better term would be a positive modifier.

Dr. Freeman: Dr. Amos, my question concerns the conclusion, or the hypothesis that there is a labile governor in the serum. When we plant primary cells, we have

an initial lag phase. It is certainly 18–24 hours. Then, we have a cell generation time of, again, a minimum of 18 hours. If we have a labile governor, it would seem that by the time the cell has completed the first generation, there is none left in the serum we have provided. Therefore, we should see at least a decline in the rate of growth. In most cell systems, in primary cells, the cells go through a normal growth curve without a change of serum.

The question I want to ask is: When you compare the same lot of serum, which has been incubated at 37 C or some other temperature for varying lengths of time, do you demonstrate a difference in plating efficiency or the growth curve or generation time in the cells within the study?

Dr. Amos: First, Dr. Freeman, I was not talking about the governor but only about positive signals in serum. I was talking about a governor inside the cells.

Dr. Freeman: But it was that the governor was triggered—

Dr. Amos: The governor was decaying. And the activators from serum would allow the protein synthesis to take place.

Dr. Freeman: If the serum is not the labile question, why are we being so careful in the processing? This is my question.

Dr. Amos: To keep a cell functioning consistently, you have to provide it with the signals it was receiving when it was inside the animal. But this is only to keep the cell in a good, healthy state. I am not talking about the fact that some cells will grow, but only about protein synthesis. My cells don't grow. They are only making protein.

Dr. Bell: I have a few questions for Dr. Amos about the character of the polysomes in the cells viewed in the serum. Although you didn't indicate it, it seems to me from your evidence that there are two classes of polysomes—one active and another inactive.

Since you do show by means of column chromatography that some proteins continue to be made during this period of relative quiescence, at least after depriving the cells of serum, can you distinguish by treatment with ribonuclease, for example, between the active and the inactive classes? Has the inactive class acquired stability as a result of its inactivity?

Dr. Amos: As a matter of fact, we didn't ask ourselves that question as perhaps we should. But we were dealing with 20% or so residual protein synthesis throughout. The fact is, they do acquire stability. Most of these polysomes are now resistant. You have to use both trypsin and ribonuclease to break them up.

Dr. Bell: Have you shown that these are not simply aggregates, nonspecific aggregates, of ribosomes, but are indeed ribosomes in association with message?

Dr. Amos: We have not shown that they are not nonspecific aggregates. The only evidence we have is really the internal evidence that you can turn them back on again in the whole cell to the extent of about 80% of their protein synthesis without RNA synthesis. I admit that the weakness there is they could be reaggregating, too.

Dr. Bell: You could be dealing with a new population of polysomes which consist of ribosomes together with—

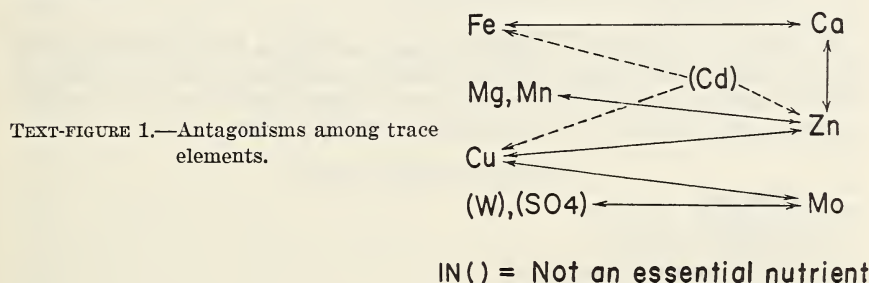
Dr. Amos: Ribosomes plus old message, yes. I couldn't distinguish.

Dr. Ling: One of the areas that has not been considered adequately in making up synthetic media is the antagonism and interaction among nutrients and also the interaction between nutrients and hormones. As cells contain multienzyme systems, their behavior or function depends on which enzymes are stimulated or inhibited. Table 1 shows examples of a number of enzymes that are activated by magnesium or manganese but are inhibited by zinc or calcium. It may be expected that the behavior of these enzymes depends on the balance between the antagonizing ions in the culture medium.

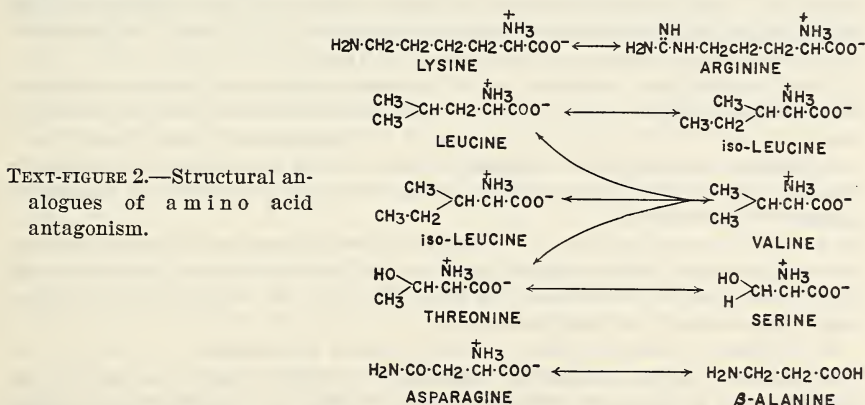
TABLE 1.—Effects of trace metal ions on enzyme activity

Enzymes	Activating	Inhibiting
Alkaline phosphatase.....	Mg ⁺⁺ , Mn ⁺⁺ , Co ⁺⁺	Zn ⁺⁺
Imidodipeptidase.....	Mn ⁺⁺	Zn ⁺⁺
Creatine phosphokinase.....	Mg ⁺⁺ , Mn ⁺⁺ , Ca ⁺⁺	Zn ⁺⁺
Adenosinetriphosphatase.....	Mn ⁺⁺ or Mg ⁺⁺	Ca ⁺⁺
Glutamine synethetase.....	Mg ⁺⁺ or Mn ⁺⁺	Ca ⁺⁺
Flavokinase.....	Mg ⁺⁺ , Zn ⁺⁺	Ca ⁺⁺
Glycyl-leucine dipeptidase.....	Zn ⁺⁺ , Mn ⁺⁺	Ca ⁺⁺

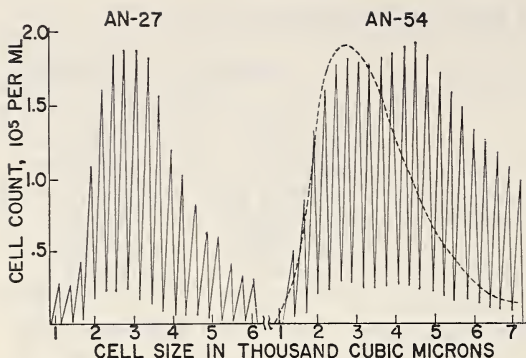
Text-figure 1 shows antagonism among the "trace" metal ions on cellular metabolism.



It has been reported that if the concentration of a single amino acid in the media is raised, it will soon reach a level showing growth inhibition or other toxic effects. However, when a suitable amount of antagonizing amino acid is supplied, the deleterious effect will not be manifested even at much higher concentrations (text-fig. 2).



In media containing amino acids in more balanced proportions, we were able to raise the total amino acid concentration 10-15 times above that of most of the chemically defined media (CDM) currently in use. Our experience with these mixtures during the past 5 years has demonstrated the beneficial effect of higher amino acid concentrations as shown in text-figure 3.



TEXT-FIGURE 3.—Amino acid concentration and cell size distribution. An-27 and An-54 contain 27 and 54 mmoles of amino acids per liter, respectively.

We have had a considerable interest in lipid metabolism. Here antagonisms exist between linoleic and linolenic acids and between arachidonic and oleic acids. Antagonism between polyunsaturated and saturated fatty acids has also been reported. Other types of interaction among nutrients are outlined below.

Interaction among nutrients

1. Chelation
2. Adsorption, carrier
3. Complex formation
4. Solubilization, precipitation
5. Oxidation, reduction
6. Interconversion, inter-dependence
7. Inactivation, neutralization, etc.

Dr. Amos mentioned the brief lifespan of hormones in relation to their action. This is important for the regulatory function of hormones in cell metabolism. The amino acid tryptophan and its metabolites, *i.e.*, serotonin, 5-hydroxytryptophan, and tryptamine will inhibit the degradation of thyroid hormone and thus prolong its action on cell metabolism. This is an example of interaction between nutrients and hormones. Interaction between vitamins and hormones is well known.

What I would like to emphasize is that we must recognize the existence of antagonisms and interactions among nutrients and between nutrients and hormones and should seriously consider such interactions in the formulation of synthetic media.

Dr. Morgan mentioned the beneficial effect of adding lactalbumin hydrolysate to culture media "that seems to stimulate cell growth even in the presence of adequate amounts of the nutrients."

I would like to point out that most of the chemically defined media contain between 125 and 150 mg of amino nitrogen per liter; 0.5% of lactalbumin hydrolysate will supply 800 mg of amino nitrogen per liter. Therefore, the difference is quite large and is not an insignificant increase.

Dr. Willmer: Would you like to comment on that, Dr. Morgan?

Dr. Morgan: In the experiments I mentioned in which the addition of lactalbumin hydrolysate to medium 199, which does have roughly the concentrations of amino acid discussed, we could demonstrate an effect from the lactalbumin hydrolysate but could not demonstrate an effect by doubling or quadrupling the level of the amino acid bloc. So, we were actually, I think, showing an effect over and above that of the individual amino acids.

Dr. Ling: I think this suggests that the balance between the various amino acids is important.

Dr. Melnykovych: Dr. Amos, have you done any work on the effect of the time of incubation on the protein profile in primary chick cells? Obviously, the withdrawal of serum would affect the position of growth rate and the production of proteins. How does this correlate with the serum withdrawal?

Dr. Amos: I haven't done those experiments, but would like to do them.

Dr. Shannon: Would you elaborate a bit on the possible reasons for the difference in behavior of the primary cells in cell suspension and on glass? To take liberty with a term used by Dr. Waymouth, I believe one could, at least in part, evoke an archaisitic interpretation of some of these differences, in that microenvironments would be more easily provided by the cells in the static culture system and more difficult to produce in cell suspension systems, at least with primary cells.

Dr. Amos: I certainly couldn't disagree with that. It is quite possible; I am using the same information to advance my own argument.

Dr. Lustroh: Several times during your talk you referred to lability of the macromolecules. Admittedly, the term "labile" is somewhat subjective. But if we could agree to define it, perhaps as lability being the loss of specific biological function with small alterations in environment such as pH or ion concentration, we could pursue it just a little further. I have no idea how many labile macromolecules are in a cell. But, with the effects of the hormones, we do have some information.

During the last few years, I have become impressed not with the lability but with the stability of the polypeptide and protein hormones. I would like to cite just one example. The molecule stimulator hormone from the anterior pituitary has a long history of being inactivated when isolated. It turns out that the molecule, in terms of the definition I have given, is not labile at all. But it apparently has been isolated in association with a protease. And the more gently the molecule was treated in its isolation, the more protease came down with it and the more degradation there was of the specific hormone. In terms of this, I think the half-life of the hormones is, indeed, short. I think we may be talking, however, about biological lability. Whether this is, in fact, related to specific enzymes, chewing them up, or whether the macromolecules themselves are labile, I think is very important.

Our experience with hormones has been that you can treat them so gently—the protein hormones, that is—that you save everything that is going to destroy them. And I don't know what you are going to do if you handle the serum very, very gently.

Dr. Amos: I think that is so well put I shan't say anything. But with insulin, also, it is clear that in the serum the destruction of insulin is by an enzyme. I was speaking not just of the molecules, their denaturation on isolation, but of their metabolic half-life in the animal. Probably there are specific enzymes in some cases which destroy them in the animal.

Dr. Paul: I believe Dr. Amos is raising the question of perhaps cell-specific hormones and even function-specific hormones. This is an idea which has waxed and waned over the years. I am very much in empathy with it. There is one example you haven't mentioned, erythropoietin, which is a very specific hormone with a very specific effect. In some embryonic liver studies we have been doing recently with people, we find that the synthesis of one of the chains of an adult hemoglobin is entirely dependent on the erythropoietin stimulus. I think this is substantial support for the ideas you are discussing.

I am interested in your suggestion that the control mechanisms here might exist at the translation level. In microorganisms, there have been a lot of ideas about charged and uncharged transfer RNA being involved in this. This has also been suggested in the control of hemoglobin synthesis. Listening to your data, it seemed to me they fitted in quite well with such a hypothesis. At least, that the internal activator might well be a charge as against uncharged transfer RNA.

Dr. Amos: I would first like to comment on the erythropoietin. It is in my printed text, but I had to skip it because of time in the presentation. The erythropoietin is

an interesting question just in general. I didn't know of the other information. I am not so sure I can see how activating enzymes could be involved. My experiments are all done adding a standard supernatant fraction that includes activating enzymes. Still, the ribosomes that one gets from the serum-starved cell do not function.

I should also answer Dr. Bell's question—it did not occur to me at the time. Even though these may be reconstructed polysomes, this also doesn't explain the unresponsiveness of these particles to exogenous messages. Dr. Bell's group has done some nice work on aggregated ribosomes and knows much more about them than I do.

I would like to comment a bit more on the activating enzymes.

Dr. Paul: Transfer RNA?

Dr. Amos: That's right, the RNA. I am not sure how that would account for any of the things I see.

Dr. Paul: I will talk about it later. Maybe I have got it wrong.

Dr. Eylar: Dr. Amos, I am struck by the analysis between the terms of the Jacob-Monod mechanism for a repressor at the DNA level and this particular governor. I gather you feel that the fact the synthesis of some protein is inhibited and some is not may indicate the presence of different classifications of polysomes. This could be explained by the amount and perhaps different kinds of governor. There is also the fact that the governor apparently does not inhibit its own synthesis. Did you examine excreted proteins and intracellular proteins?

Dr. Amos: No, we haven't thought about the governor inhibiting its own synthesis. Of course, when we use the word "governor," we consider that there may be several kinds and classes of "governor."

The other thing of interest is that we have looked at L cells to a reasonable degree and at HeLa's to a limited extent. We didn't find any overshoot in L cells. We think they have lost their governor molecules. You can't get any overshoot when you shut them off. They will grow without serum and multiply perfectly well. We take ordinary glass, expose it to serum, wash away the serum, and plant the L cells on it. They grow very nicely without serum. But when you give them serum, you still turn on proteins they don't normally make and don't need. They still have the memory that certain positive signals can turn on.

The only point I really wanted to make is that I think one finally explains it, either at the level of DNA or at the level of polysomes, by the fact that the cells have been selected for responding to positive signals.

Dr. Eylar: They could be making their own repressors, too.

Dr. Amos: Repressors, yes. They are certainly full of the repressors of the Monod-Jacob type. Yes, they have repressors, whatever these may be.

CELLULAR FINE STRUCTURE

Chairman: Dr. Keith R. Porter, Harvard University, Cambridge, Massachusetts.

Speakers: Dr. Eugene Bell, Massachusetts Institute of Technology, Cambridge, Massachusetts

Dr. Jerry S. Sutton, Johns Hopkins Medical School, Baltimore, Maryland

Panelists: Dr. Albert J. Dalton, National Cancer Institute, Bethesda, Maryland

Dr. Joseph G. Gall, Yale University, New Haven, Connecticut

Dr. Don W. Fawcett, Harvard Medical School, Boston, Massachusetts

Dr. Porter: The title of this session is, I believe, slightly mispointed. As I looked over the program and the content of the talks to be given, I realized that actually the discussion is focused very largely on the phenomena of cell differentiation. To some extent, these phenomena will be presented in terms of cell fine structure, especially by Dr. Sutton. Nonetheless, this is done with the idea of depicting for you the transformation of one cell type or the changes as one cell type changes from one form to another.

Dr. Bell is more interested in the genetic control of protein synthesis, especially as this finds expression in early development. Thus, we may learn from him how some parts of the genetic information are replicated and transcribed into mobile messengers and how others are repressed or held in abeyance to be later activated in some sequence consistent with the production of what the cell and organism need for their differentiation.

Control of Synthetic Activity in Differentiating Cells^{1, 2}

EUGENE BELL, *Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

SUMMARY

Problems of gene activation and transcription, gene repression, messenger RNA synthesis and transport, and control of gene expression at the level of translation during development are reviewed.—Nat Cancer Inst Monogr 26: 51–70, 1967.

PRESENT VIEWS of the sequence between gene activation and the appearance of protein products may help direct attention to problems of macromolecular synthesis that seem significant for an understanding of development.

GENE ACTIVATION AND TRANSCRIPTION

Little is known about how gene transcription is initiated or how the rate of transcription is changed, but the time of occurrence of gene activation can be inferred from the appearance of ultimate products. Such information is subject to the limitations of technique upon which detection of gene products depends.

Although one cannot be certain that a very large number of genes in differentiating cells is not leaky, *i.e.*, being transcribed slowly, examples can be presented which satisfy the general condition of activation from a rate of transcription = 0 to a rate which is >0 .

The appearance of thymidine kinase before the beginning of DNA synthesis in *Lilium* microspores, which are antecedents of pollen, is an example of initiation of genic activity during development. Appropriate metabolic inhibitors were used to show that synthesis of enzyme

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

² Supported by grant 614 from the National Science Foundation.

depends on synthesis of RNA and that enzyme activity, when it appears, is associated with newly made protein (1).

Another example of appearance of an enzyme during the developmental sequence is provided by studies on slime mold (2). UDP-galactose polysaccharide transferase, which is absent in vegetative cells, appears when the pseudoplasmodium comes to rest. *De novo* appearance of the enzyme is preceded by RNA synthesis. Enzyme is lacking in morphologically deficient mutants that cannot synthesize the structural mucopolysaccharide for whose assembly enzyme is needed (3).

It is somewhat more difficult to fix the origin of function of genes that code for structural proteins, since assays for them are less sensitive than those for enzymatic proteins, but at least one example is available. The final transcription required for an erythrocyte bearing hemoglobin occurs just before headfold stage in the chick embryo (6). If RNA synthesis is blocked much before that time, hemoglobin is not made.

Other appearances of structural proteins during development can be cited, but without evidence that they reflect immediately preceding gene activity or that they don't result from assembly of previously synthesized subunits.

Another general category of activation at the level of DNA is gene stimulation which results in an increase in the rate of transcription. The thousandfold increase per cell of pancreatic amylase in acini of the developing pancreas is an illustration of stepped up gene function (4). RNA synthesis is required for the appearance of morphologically differentiated pancreatic cells in which the increase of enzyme occurs (5).

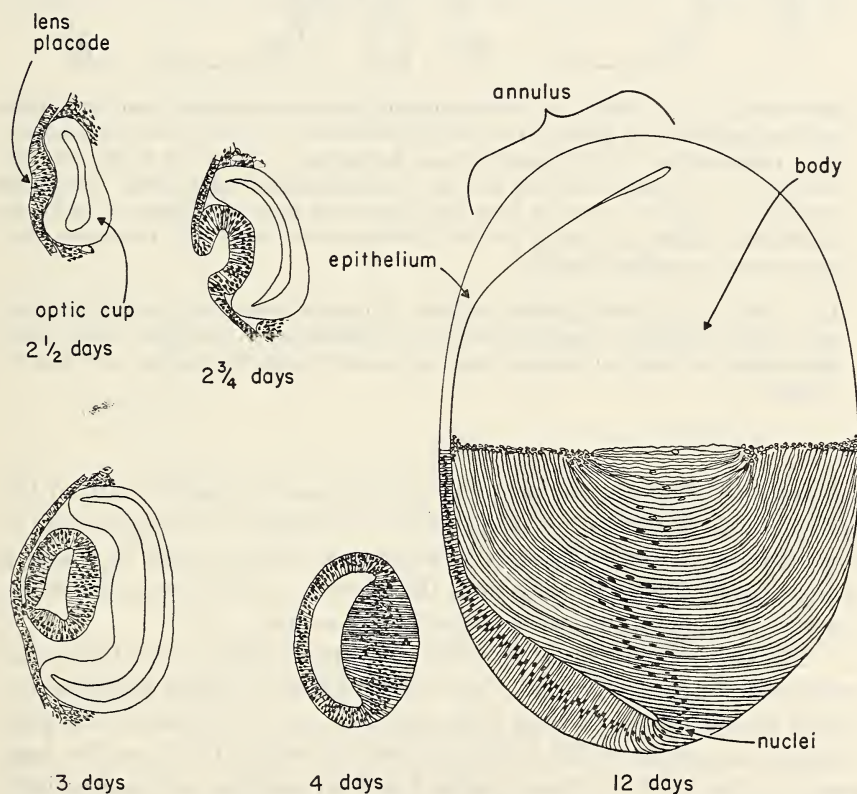
When embryonic chick retina is explanted to tissue culture, a marked increase in the activity of the enzyme glutamine synthetase is recorded. The increase does not occur in the absence of RNA synthesis and appears to be owing to *de novo* synthesis of protein (7). An increase in enzyme activity need not reflect gene activation. This is clearly shown now with tryptophan pyrrolase, whose synthesis was thought to increase by substrate induction (8). The effect is due to stabilization of enzyme by substrate, but an increase in the rate of synthesis of the enzyme arising from *de novo* synthesis can be triggered by corticosteroid hormones. In fact Amos has now shown that the increased rate of synthesis of glutamine synthetase in explanted retinas is due to the presence of hormone in the horse serum used as incubation medium.

GENE REPRESSION

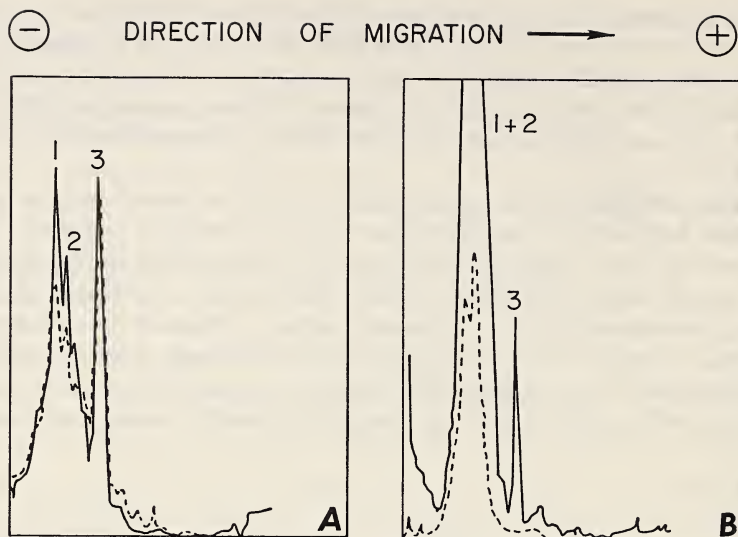
Probably equally important for developmental sequences is the deactivation or repression of genes, which results in complete cessation of transcription or in a reduction of the rate of transcription. Trivial mechanistically, but significant for development, is the device of nuclear ex-

trusion in the erythrocyte. It effectively deactivates all nuclear genes. In some erythrocytes, *e.g.*, those of the bird, all genes are expressed by a mechanism yet unknown, without nuclear extrusion. The results are similar. The previous products of a few genes, stabilized messenger RNA (m-RNA) for hemoglobin, can function without competition for a long period.

In the developing lens, deactivation of genes occurs at least twice as germinal cells of the lens epithelium migrate through the annulus into the lens body and elongate (text-fig. 1) (9). Cells of the epithelium produce over 20 electrophoretically identifiable proteins (text-fig. 2A), including those made in the annulus and the body. While in the annulus, the transitional zone between the epithelium and the body, and in the body, the cells cease to make all proteins except two (text-fig. 2B). In the annulus and body, cells undergo striking elongation, possibly mediated by micro-



TEXT-FIGURE 1.—Morphological development of the chick lens. After the initial period of development, at about 4–5 days, the anterior epithelium becomes the germinal source of new lens cells. Divisions occurring there give rise to cells that move through the annulus and into the lens body. During the process cells undergo extensive elongation.



TEXT-FIGURE 2.—(A) Stain and autoradiograph patterns obtained from acrylamide gel electrophoresis of protein from the epithelium of the 12-day chick lens. Lenses were pulse-labeled with ^{14}C -algal protein hydrolysate. About 20 bands are discernible in the stain gel and all have acid-insoluble radioactivity associated with them, which indicates at least that number of proteins is being made in the epithelium. Bands 1, 2, and 3 are the principal ones detected in the whole lens. All are made in epithelial cells.

(B) Stain and autoradiograph patterns of protein from the 12-day chick lens body. Only 2 proteins (bands 1 and 2) are synthesized in the body, even though many others are carried there in cells that move through the annulus and begin to elongate.

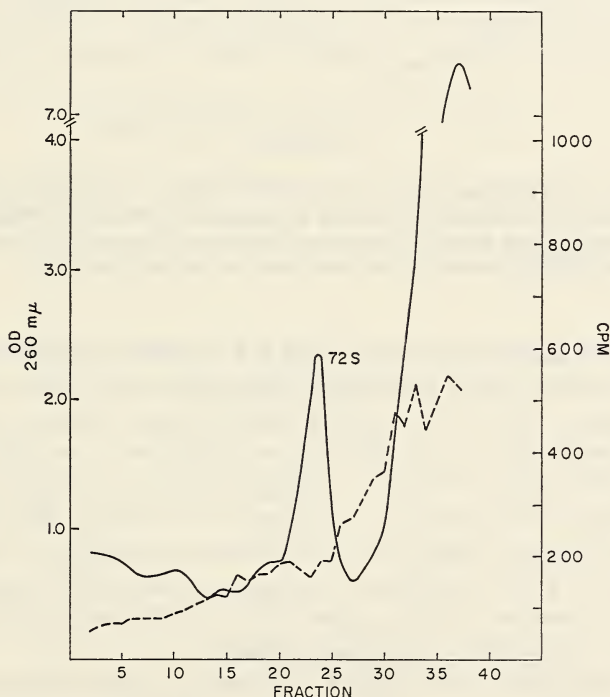
tubules (10). Perhaps the principal protein made while cells are in the epithelium is the protein for microtubules. This protein is not produced in the body but is carried there with migrating epithelial cells in which it is not degraded. In the body cells, the protein could be utilized for the extension of existing microtubules or for new ones.

The two proteins made by body cells are probably crystallines, and eventually fill the body cells. Gene transcription for those proteins continues for some time in body cells and then ceases. The immediate gene product, long-lived m-RNA for the body proteins, functions for long periods after RNA synthesis stops. The final cessation of gene activity seems to be correlated with nuclear disintegration (11).

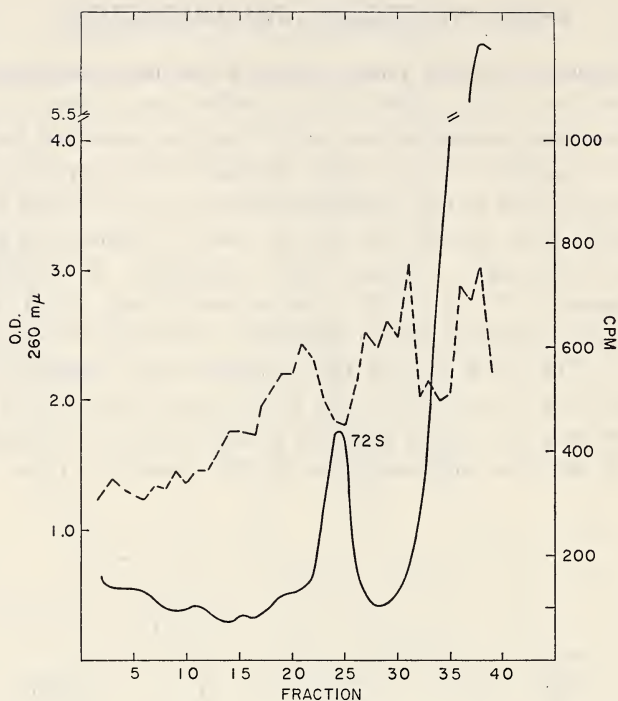
It remains to be shown how most of the genome in lens cells can be repressed while a few cistrons continue to function and then how the final few are repressed.

m-RNA SYNTHESIS AND TRANSPORT

The transport of m-RNA from nucleus, where most templates for protein synthesis are made, to cytoplasm is another step in the sequence from gene to specialized product whose control may be important for development. We have studied the movement through various cytoplasmic pools of newly made RNA in the developing feather. After 12-day embryonic chick feathers were labeled for 10, 25, and 40 minutes *in vitro* with ^3H -uridine, cytoplasmic extracts were displayed in 15–30% linear sucrose gradients (12–14). In 10 minutes newly made RNA was recovered free in the cytoplasm and in association with 40S ribosomal subunits (text-fig. 3). The RNA free in the cytoplasm was largely 4–6S RNA and was probably transfer RNA. By 25 minutes, incorporation of radioactivity into both 40 and 60S particles (possibly ribosomal subunits) and in a pool of particles sedimenting at 85–90S (text-fig. 4) was observed.



TEXT-FIGURE 3.—Twelve-day feathers were labeled for 10 minutes with 100 $\mu\text{c}/\text{ml}$ ^3H -uridine. A cytoplasmic extract was displayed in a 15–30% linear sucrose gradient and spun for 5 hours at 24,000 rpm in a Model L Spinco. Acid-insoluble radioactivity (dashed line) sediments in shoulders at 60S and 40S and ascends to the top of the gradient.



TEXT-FIGURE 4.—Cytoplasmic extract from 12-day feathers was labeled for 25 minutes with 100 $\mu\text{c}/\text{ml}$ ^3H -uridine. In addition to peaks of radioactivity, which sediment with particles lighter than 72S ribosomes, acid-insoluble counts are observed in a peak at 85–90S and descend to the heavy end of the gradient.

At 40 minutes (text-fig. 5) newly made RNA predominated in the 85–90S pool and was observed in association with polysomes as well as with ribosomal subunits. The RNA associated with ribosomal subunits, which was made during 40 minutes of labeling, was polydisperse and was predominantly nonribosomal (text-fig. 6). I suggest that it was largely m-RNA. Little difference was observed in the character of RNA made during 40 minutes of labeling, when protein synthesis was brought to a halt with actidione. The pool of ribosomal subunits available for transport of m-RNA must, therefore, be large, since there is little change in the kinetics of transport in the absence of protein synthesis.

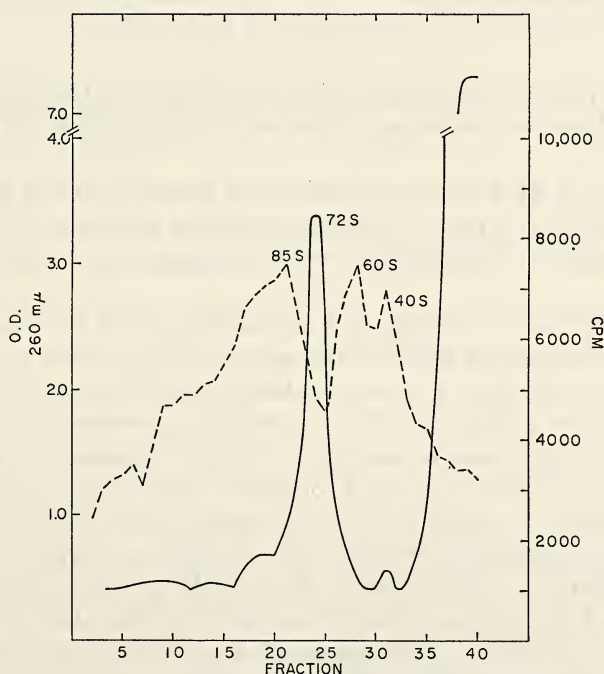
It can be proposed that m-RNA becomes associated with either 40 or 60S ribosomal subunits (or other particles) and in that form is transferred to an 85–90S pool of particles. The particle could consist of a 40 or 60S ribosomal subunit with m-RNA in combination with a 72S ribosome. The particle, after accepting the missing subunit, would take additional ribosomes as it begins to function in protein synthesis.

In a search for mechanisms at the translational level through which gene expression might be controlled, steps involving movement of m-RNA on precursor particles, which themselves cannot translate until they are saturated, require scrutiny. Messenger RNA for future use could be stored on an unsaturated ribosomal subunit or in a 90S particle such as the one described.

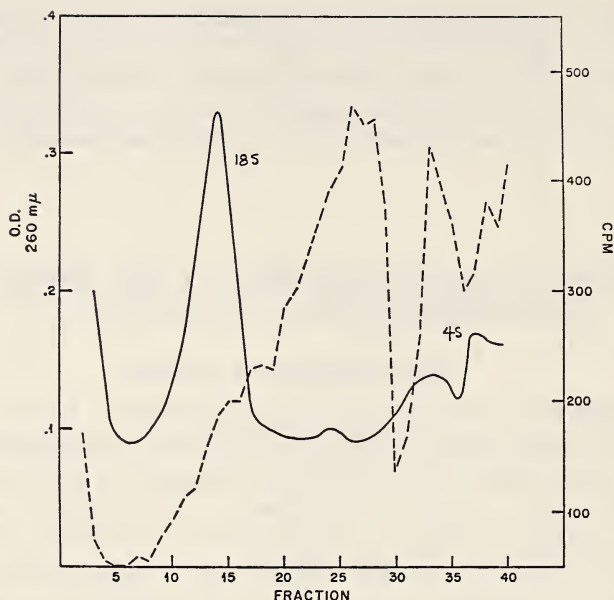
CONTROL OF GENE EXPRESSION AT THE LEVEL OF TRANSLATION DURING DEVELOPMENT

Characteristics of m-RNA

Messenger RNA of both long and short half-life is found in higher cells (15). We have shown that they coexist in the developing chick lens (text-fig. 7) (16). Recently we presented evidence (17) that they coexist in the developing 11- and 12-day feather in which they are regionally segregated. Whereas m-RNA of long half-life is localized primarily in the cells of the 12-day feather sheath, messenger of short half-life is found throughout the remainder of the feather and possibly in the sheath as



TEXT-FIGURE 5.—Twelve-day feathers were labeled for 40 minutes with ^3H -uridine. The peak at 85S is equal in height to the peaks at 40 and 60S.



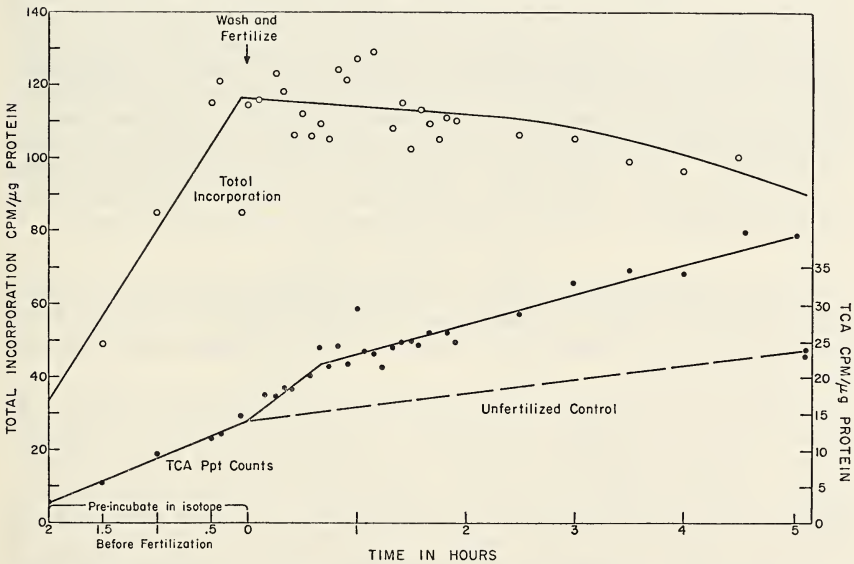
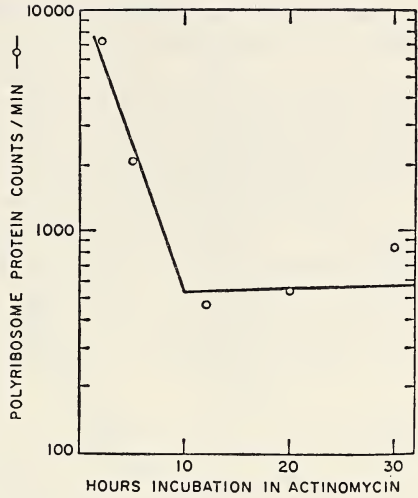
TEXT-FIGURE 6.—Sedimentation analysis of RNA taken from the 40S region of a 15–30% linear sucrose gradient in which a cytoplasmic extract was displayed. Feathers were labeled for 40 minutes with 100 $\mu\text{C}/\text{ml}$ ^3H -uridine.

well (fig. 1). We have no firm evidence yet regarding the coexistence of long- and short-lived messages in the same cells in either the feather or the lens.

Long-lived m-RNA can be synthesized for immediate use or for storage. In the developing retina, the enzyme glutamine synthetase is translated from a message of long half-life, but the message is made for immediate use (7).

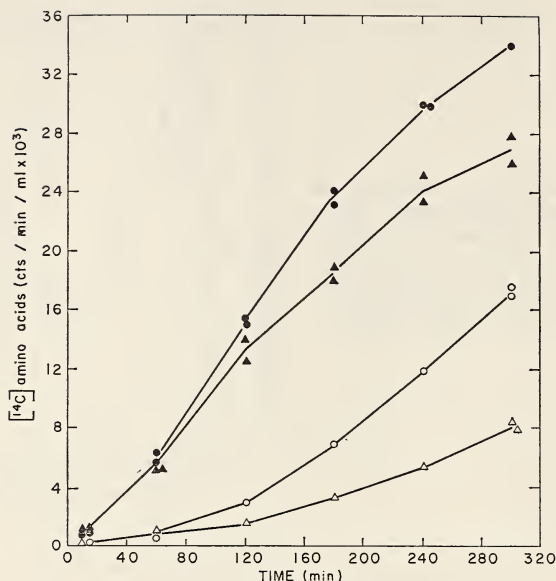
On the other hand, message of long half-life stored for use at a later stage of development is found in the eggs of the sea urchin (18) and the clam (19). In text-figure 8 protein synthesis is shown in control clam eggs before and after fertilization. For the first 5 hours after fertilization the rate of protein synthesis is unaffected if eggs are pretreated for 4 hours with 25 $\mu\text{g}/\text{ml}$ actinomycin D. At a concentration of 50 $\mu\text{g}/\text{ml}$, actinomycin D has no effect on protein synthesis in the unfertilized egg. It begins to affect fertilized eggs, only partly, about 1 hour after fertilization (text-fig. 9). Cleavage in eggs treated with 25 or 50 $\mu\text{g}/\text{ml}$ actinomycin D is inhibited and grossly abnormal, which indicates that the antibiotic has no difficulty in penetrating the egg membranes. In fact, eggs treated with either 25 or 50 $\mu\text{g}/\text{ml}$ actinomycin D do not ordinarily cleave more than a few times.

TEXT-FIGURE 7.—Decay of polysomes in 12-day lens cells. The biphasic character of decay suggests two populations of messenger RNA: one with a half-life of about 3 hours and the other with a considerably longer half-life.



TEXT-FIGURE 8.—Mature, unfertilized clam eggs were labeled for 2 hours with 10 $\mu\text{C}/\text{ml}$ ^{14}C -amino acids (Schwartz RPH) and samples were taken at intervals for determination of total incorporation; for acid-precipitable radioactivity and Lowry protein. Eggs were then washed, fertilized, and allowed to develop to hatching (free-swimming blastula stage). During the 5-hour period, samples were taken as before fertilization and from an unfertilized control group. *Note* that during the experiment there is little loss in endogenous labeled pool as reflected by total incorporation.

CELL TISSUE AND ORGAN CULTURE



TEXT-FIGURE 9.—Eggs were treated for 4 hours with 50 $\mu\text{g/ml}$ actinomycin D prior to fertilization. After they were fertilized and incubated with 5 $\mu\text{c/ml}$ ^{14}C -algal protein hydrolysate, protein synthesis (cpm/mg protein) was determined at intervals. For the 1st hour no difference between control eggs not treated with actinomycin D and experimental eggs is seen. By 5 hours, a 50% reduction in protein synthesis is observed in actinomycin-treated eggs. At 25 $\mu\text{g/ml}$ no reduction in protein synthesis is noted, but either 25 or 50 μg interferes with normal cleavage so that no more than a few cell divisions occur. ●—● Total incorporation, control; ○—○ acid-insoluble incorporation, control; ▲—▲ total incorporation, actinomycin D-treated; △—△ acid-insoluble incorporation, actinomycin D-treated.

We have concluded, with caution, that there is sufficient template stored in the unfertilized egg to mediate protein synthesis in the absence of RNA synthesis without a reduction in synthetic rate, at least until hatching takes place at 5 hours.

We have not yet shown, either in the clam or in the sea urchin, that the rate change in protein synthesis for which fertilization is responsible is accompanied by the synthesis of proteins not made in the unfertilized egg. This means that m-RNA may not be stored at all but used at different rates in the unfertilized and fertilized egg. We now have evidence in both the clam and the sea urchin that the mature unfertilized egg is actively engaged in protein synthesis (19, 20), and that fertilization does not initiate protein synthesis but only stimulates a change in its rate. In the clam the rate change is of the order of 4, in the sea urchin, *Arbacia punctulata*, it is 4, and in another species of sea urchin *Strongylocentrotus purpuratus*, the increase after fertilization is about 30-fold.

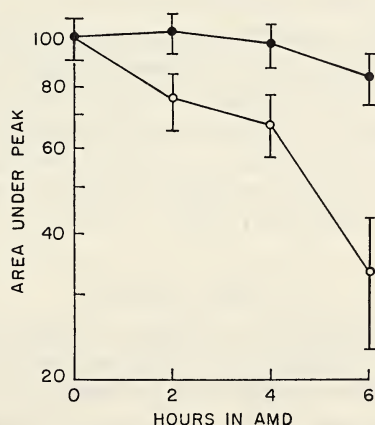
If the kinds of proteins made after fertilization are not different from those made before fertilization, then the egg may not store messages of long half-life but simply translate them less frequently than after fertiliza-

tion. If entirely new proteins are made as a consequence of fertilization, then either m-RNA is stored in the egg or newly made m-RNA is responsible for their appearance after fertilization.

It has been suggested, but not proved, that m-RNA made during the cleavage stages of sea urchin development is not put into use until some time after hatching (21). If correct, this would constitute another example of storage of m-RNA for use during another phase of development.

Whether short-lived messages can be made for storage as well as for immediate use is yet unanswered. Messenger RNA for the enzyme UDP-galactose polysaccharide transferase, to which I have already alluded, appears to be made in advance of its use (3), but kinetic studies have not yet been done to tell whether the half-life of the message is greater than that of other m-RNAs in the slime mold.

There is no definitive information on whether m-RNA for the same protein can be both short-lived and long-lived. In our work on the lens there is a suggestion that it can. While cells are in the epithelium, at least one protein (text-fig. 10) and probably all detectable by electrophoresis (22) are synthesized on m-RNA of short half-life. The two proteins made by cells in the lens body are also produced while cells are part of the epithelium. After becoming part of the body, the same proteins are made on m-RNA of long half-life (text-fig. 10) (9). The result is subject to the reservation that, while identification of long-lived m-RNA through use of actinomycin D has validity, similar identification of short-lived m-RNA rests on less firm ground, because of the various side effects of the antibiotic and because of the possible differential susceptibility to side effects of actinomycin D of different parts of the same organ.



TEXT-FIGURE 10.—Effect of actinomycin D (AMD) on synthesis of proteins of the lens body and on synthesis of one protein of the lens epithelium. Soluble protein from lenses was electrophoresed and the specific activity of each band determined (19). Specific activities are plotted as percent of the initial value. *Top curve* is decay of body proteins; *bottom curve* is decay of epithelial proteins.

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The Ribosome

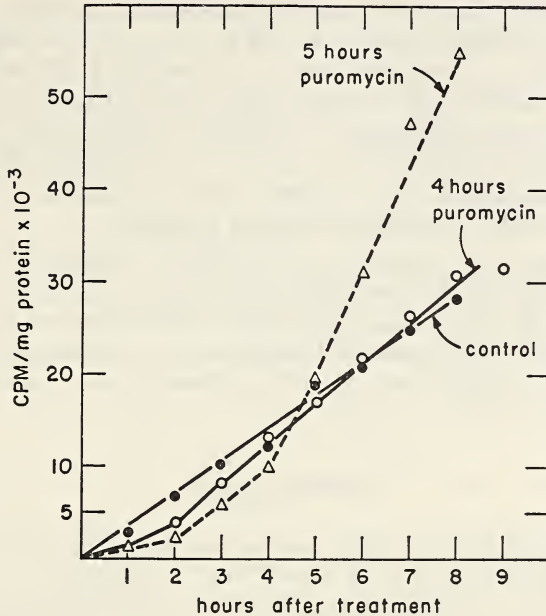
It is not clear how m-RNA may be stored or through what mechanism its use is controlled; however, there have been some attractive possibilities. Monroy *et al.* (23) have reported that ribosomes from the unfertilized sea urchin egg cannot mediate protein synthesis in a cell-free system. If these ribosomes are treated with low concentrations of trypsin, they are activated, and without the addition of exogenous message function, to make protein in a cell-free system. The results suggest that the repressed ribosome of the unfertilized egg is equipped with a molecule of m-RNA. Possibly, the cortical reaction which accompanies fertilization of the sea urchin egg releases proteolytic enzymes responsible for the activation of cytoplasmic ribosomes. An interesting corollary has been reported in the work of Salb and Marcus (24). They showed that, during mitotic metaphase of a synchronously dividing population of HeLa cells, ribosomes from the cells cannot function in a cell-free system. However, mild tryptic digestion renders them functional.

There seems little doubt that ribosomes can be specially adapted for developmental requirements. In adult clam tissues, only 72S ribosomes are found, yet in the mature unfertilized egg of the clam ribosomes are predominantly 80S. The characteristics of egg and adult ribosomes have been compared and the S values of low magnesium subunits and of 0.5 M NaCl-treated ribosomes are lower for adult as compared with embryonic particles. The 80S ribosome of the unfertilized clam egg may be a repressed ribosome.

It is probable that two populations of ribosomes coexist in the unfertilized egg of the clam (19). One is a population of active ribosomes (72S) which engages in protein synthesis and the other a population of large ribosomes (80S) which appears to be repressed. Following fertilization there is a continuous increase in the rate of protein synthesis and in the appearance of functional polysomes (19). These facts, as well as the absence of 80S ribosomes in adult tissues, have led to the suggestion that ribosomes are activated gradually following fertilization, and that the process of activation occurs throughout the period of cleavage. How the 80S ribosomes are transformed to 72S ribosomes is a question for further study.

Protein Synthesis

In exploring the significance of protein synthesis in the mature unfertilized egg, for later development, we have used two different inhibitors of protein synthesis (20): a specific one, puromycin, and a nonspecific one, nitrogen. Eggs of the sea urchin *A. punctulata* were put into sea water containing 1 μ C/ml RPH (Schwartz: a mixture of labeled amino acids obtained from hydrolyzed yeast protein) following 4 hours in sea water with or without 235 μ g/ml puromycin or 5 hours in sea water with puromycin in the same concentration. TCA-precipitable cpm/mg protein was



TEXT-FIGURE 11.—Unfertilized *Arbacia punctulata* eggs were put into sea water containing 1 μ /ml Schwartz reconstituted protein ^{14} C-hydrolysate (RPH) following 4 hours in sea water with (○) or without (●) 235 μ g/ml puromycin or 5 hours in sea water with puromycin in the same concentration. TCA-precipitable cpm/mg protein was determined at hourly intervals thereafter. While 4 hours in puromycin was insufficient to stimulate protein synthesis, after removal of the inhibitor 5 hours was sufficient.

determined at hourly intervals thereafter. Results are shown in text-figure 11. At 5 hours after removal from puromycin, eggs treated for 5 hours with the inhibitor began to exceed controls in their rate of protein synthesis. Eggs of two species *S. purpuratus* or *A. punctulata* behaved similarly. After removal of eggs from a condition of anoxia (exposure to nitrogen), a several-fold enhancement of protein synthesis was also observed.

The rate of protein synthesis in the unfertilized sea urchin egg appears to be strictly regulated. As in the clam, it is unchanging for a long period and is several times lower than the rate during the 1st hour after fertilization. To cause a stimulation of protein synthesis, by first blocking it and then releasing it, would suggest that regulation depends partly on a product of protein synthesis itself.

But the increase in synthetic rate seems to be due to two separate mechanisms, one of which is mimicked by puromycin or anoxia and the other reproduced by removal of CO_2 from the sea water by treatment with pure O_2 or a mixture of N_2 and O_2 . Synthesis in *A. punctulata* is

increased twofold and in *S. purpuratus* 20-fold by removal of CO₂. The two, exposure of eggs to an inhibitor of protein synthesis and subsequent removal of CO₂, act to elevate protein synthesis to a rate approximately equal to that achieved in the 1st hour after fertilization. Subsequent fertilization of eggs stimulated in this way did not lead to additional increase of protein synthesis. Removal of CO₂ (or addition of O₂), though, may affect the rate and level of protein synthesis by a route not in direct communication with way stations of gene expression.

Any scheme for the regulation of the level of protein synthesis in the unfertilized egg would be required to take into account the mechanism employed for the repression of the bulk of the ribosomal population as well as the factors just discussed. How these control processes are modified at the time of fertilization and during cleavage is a question of basic interest.

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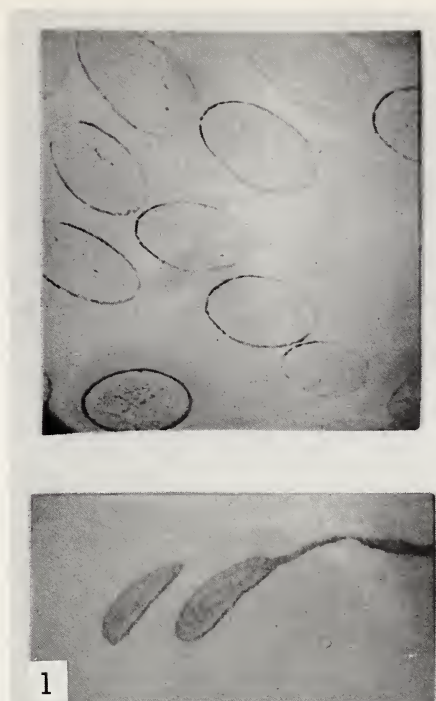


FIGURE 1.—Eleven-day feathers were incubated with 60 $\mu\text{g}/\text{ml}$ actinomycin D for 24 hours and then pulse-labeled for 2 hours with ^{14}C -amino acids [Schwartz reconstituted protein ^{14}C -hydrolysate (RPH)]. Autoradiograph of treated feathers gives evidence of protein synthesis in the sheath cells primarily. In control sections (*below*), synthesis occurs uniformly throughout the feather.

DISCUSSION

Dr. Porter: I am going to ask Dr. Gall to discuss some points pertaining to Dr. Bell's presentation.

Dr. Gall: Rather than direct my comments specifically to things that Dr. Bell said in his paper, I will use his very fine presentation of information on messenger RNA synthesis and ribosome formation during ovogenesis and early embryogenesis as a springboard for the comments I want to make dealing specifically with the question of ribosome RNA synthesis during ovogenesis.

To put this a little more in the general framework, the things that have been said, the discussions of Dr. Bell as well as of Dr. Amos, and Dr. McCarty's comments as a discussant, have all stemmed pretty much from the gene sequence developed from studies on *Escherichia coli* whereby the gene DNA makes or is responsible for the formation of RNA, messenger RNA, which is then involved in protein synthesis and in transferring this scheme-derived microbial system to higher organisms. Obviously there are possible control mechanisms at the level of gene transcription which, of course, is messenger RNA synthesis and at the level of the translation of the messenger RNA into protein at the polyribosomal level.

I think it is undoubtedly certain with the present various studies on embryogenesis that control of various processes, enzyme synthesis, and so forth, which Dr. Bell discussed, certainly takes place at these levels in embryonic systems. And much of differentiation is certainly based on this type of control mechanism.

However, I would like to emphasize the possibility that there may be changes in the genetic system itself during embryogenesis—not in the more classical idea of direct mutation during embryogenesis but, specifically, there may be differential replication of parts of the genome as a mechanism of long-term control or at least as a contaminant of more stable long-term types of differentiation.

I want to relate this to the question of ribosomal RNA synthesis during ovogenesis. The notion that there may be extra DNA synthesized at different times in the life of various kinds of cells has been tossed about a good deal in the last 10 years. It sometimes goes under the name of metabolic DNA and, in general, hasn't been much considered as a mechanism or the concomitant of differentiation for a couple of reasons: first, the types of systems in which it has been studied. It has not generally been possible to pin down any specific extrasynthesis of DNA, any specific process, any specific enzyme formation, or what have you. Second, the best case of differential DNA synthesis so far reported occurs in the salivary gland cells of the mycetophilidae (family) *Sciara* (species), and these cells are destined to differentiate shortly after the stage in which they are being studied. Thus it has probably been thought this mechanism might be a very special one that is not related to any gene phenomena.

With these introductory comments, I suggest that during ovogenesis the enormous amount of ribosome RNA synthesized is accompanied by differential replication of the ribosomal cistrons within the nucleus, and that at least part of the control of this increase of one particular type of RNA synthesis is not just the speeding up of the activity of the few cistrons already present, but actually involves the formation of manifold production of the basic ribosomal cistrons themselves.

To illustrate this, I shall discuss the situation in the nucleolus in the amphibian oocyte. There is a large nucleus, but around the periphery are many objects, in the past called "nucleoli." In a high magnification, autoradiography of the nuclear body, the cytoplasm, and nucleus may be seen.

The large nucleoli, after uridine incorporation, show the synthesis of RNA taking place. Morphologically, electron microscopy shows these are typical nucleoli with the differentiation into granular fibrous components. These are completely extra-chromosomal in the sense they are not attached to the chromosomes of the nucleus.

Low magnification radiomicrography of the large lampbrush chromosome pairs picks up a few of the extrachromosomal nucleoli from one nucleus.

As a matter of fact, there are approximately 1,000 nucleoli in these ovocyte nuclei—in each one, that is. This has been known for a long time, of course. And it has been equally thought that these nucleoli were simply some product of the chromosome or product of the nucleus. As long as that was the general notion, their value, I think, was somewhat secondary. But I suggest that each of these nucleoli, as a matter of fact, contains the ribosomal cistrons for the production of ribosome RNA. That is, they contain a small amount of DNA homologous to the segment which is also present on the chromosome. Thus these nucleoli have been produced by some sort of differential replication of the DNA during ovogenesis and this is a mechanism for increase in the cell's synthetic capacity.

To make this bold statement stick, it would be necessary to prove four things about these nucleoli. First, that it is legitimate to call them nucleoli. This is essentially a morphological question showing that they are homologous in a classical morphological sense to the nucleolus which is located on the chromosome. Second, we would have to show there is DNA in them. Third, we would have to show they are capable of forming ribosome RNA, and, therefore, that the DNA which is synthetically active is, in fact, ribosome cistrons. And fourth, if we were exceedingly lucky, we should demonstrate by some technique such as DNA-RNA hybridization that DNA is, in fact, ribosomal cistrons.

The first of these three has been shown. And that is essentially what I want to say. Briefly, there is one chromosome of the set which bears objects morphologically identical to the extrachromosomal nucleoli and these objects are at the locus on a particular chromosome at which the somatic nucleolus is found. This is rather old work now, but establishes that in some sense these objects are the homologues of the normal nucleoli.

A very interesting sequence of transformation has been recently described by Kalen. These spherical nucleoli, ordinarily seen in the preparation at one stage during ovogenesis, undergo a transformation from a sphere to a beaded necklace that is in the form of a circle. This occurs regularly at a particular time in ovogenesis. The interesting thing is that the ovocyte chromosome bears a spherical nucleolus. At the same time, the nucleolus on the chromosome undergoes the transformation into a circular ring form, and in doing so, it transforms itself into essentially what we call in chromosome terminology a lumpy loop.

The highly significant thing about this transformation, shown recently by Keeser and by Miller independently, was that deoxyribonuclease applied to a fresh preparation caused fragmentation of this nucleolus locus pair of loops and of the other loops, as was well known previously, and also caused fragmentation of the extrachromosomal nucleoli. Thus the fact is established that there is DNA in these structures.

Since time is limited, I won't go into the evidence that the nucleoli in these cells are actually making ribosome RNA, but this has been well established by standard biochemical techniques. I think that the conclusion is almost inevitable: Somehow this cell has undergone a differential replication of the ribosome cistrons. These have come off the chromosome, or perhaps an original copy has come off the chromosome, and replicated in the nucleus.

This may be a more general mechanism for cellular differentiation. In cases where a cell has a large job to do over a long time, it may not do it just by speeding up the rate at which translation or transcription occurs, but it may, in fact, produce extra copies of the gene which come off of the chromosomes and, therefore, do not permanently alter the genome.

Dr. Porter: It seems to me that this complicates the story considerably. Why is the DNA required for the physical integrity of the extra nucleoli? You said that deoxyribonuclease brings about their disruption.

Dr. Gall: I wouldn't phrase the question that way. DNA does form the physical structure of all the loops of the chromosomes. This demonstrates that it also forms the basis of the extrachromosomal nucleoli. It simply reinforces the homology between these structures and normal loops, which is the normal way in which the chromosome is making RNA. And, of course, it serves the very useful function of demonstrating the DNA, which is otherwise in such low concentration that it can't be demonstrated by typical cytochemical techniques.

Dr. Bell: Is there anything left to the nucleolus if you treat it with a base or ribonuclease after having previously labeled it with thymidine? Can you see foci of incorporation?

Dr. Gall: Let me answer that in two stages. If you treat the nucleoli with ribonuclease, there is essentially no change. That is, they do not break their linear continuity. Protease slowly digests them, but they always maintain the ring form. In other words, it doesn't break the backbone for unknown reasons.

Now, with these nucleoli, we have not been able to demonstrate thymidine incorporation into the DNA as yet. And I think this is probably because we haven't caught the right stages. The nucleoli, all thousands of them, are formed quite early in oogenesis and perhaps during a relatively short period.

Dr. Porter: While we are on this topic of DNA and RNA replication, could I have some questions from the floor directed specifically to Dr. Bell?

Dr. McCarty: I would like to ask Dr. Bell how he is able to differentiate the mature sea urchin egg from an immature sea urchin egg. Because if one has immature sea urchin eggs, this may account for some of the protein synthesis.

Another question is: If you break up the 85S ribosome particle and examine the RNA on a density gradient, do you find 18-28 in the normal proportion you would find in a single ribosome or is the contribution from either the original 40S particle or the original 60S?

Dr. Bell: First, the process of procuring eggs by gravity settling and taking only the rapidly settling eggs gives assurance that the eggs taken are mature. Secondly, if the eggs are 100% fertilizable they are clearly mature. Thirdly, by labeling with a protein precursor by means of autoradiography, it is possible to tell whether all eggs are synthetically active. Our experiments indicate that they are.

I can't answer your second question because we have not yet taken the cut between 85 and 90S for extraction and sedimentation analysis of the RNA made during a brief period of labeling.

Dr. Porter: Are there any other questions?

Dr. Amos: In fact, two questions. First, in the unfertilized egg, are you making only this special protein that you spoke about? Second, what happens to the future development of the egg after the puromycin treatment?

Dr. Bell: There are many proteins being made in the unfertilized egg. We don't know yet whether the proteins made after fertilization are different from those made before or whether the spectrum of proteins made changes after puromycin, nitrogen, or oxygen stimulation. These questions are being studied currently by Mr. R. Mackintosh in my laboratory.

Let me now answer your second question. After depression of protein synthesis with nitrogen, a stimulation of protein synthesis is observed after the nitrogen is washed out. Development is perfectly normal and the embryos hatch. After puromycin treatment, the eggs develop as far as hatching and will not develop thereafter.

Ultrastructural Aspects of *In Vitro* Development of Monocytes Into Macrophages, Epithelioid Cells, and Multinucleated Giant Cells^{1, 2}

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SUMMARY

Monocyte transformation into macrophages, epithelioid cells, and multinucleated giant cells in tissue culture demonstrated the following ultrastructural alterations. Cell volume increased progressively. All stages possessed abundant, highly active cytoplasm with elaborate development of cytoplasmic organelles involved in protein synthesis and concentration. In the *nucleus*, chromatin margination, pronounced in monocytes, diminished in later forms. Multiple nucleoli appeared. *Lysosomes* increased in size and number before phagocytosis. After phagocytosis a slight reduction was observed. Following digestion of phagocytosed material, lysosomes vastly increased in number and size in epithelioid and giant cells. Lysosomes fused with and discharged their contents extracellularly and into phagocytotic vacuoles. All stages possessed large quantities of *rough ER*, *smooth ER*, and free *ribosomes*. The *Golgi apparatus* (small in monocytes) increased in size and complexity. Its ultrastructure in macrophages and epithelioid cells suggested

that lysosomes form there. In giant cells, Golgi regions were small and multiple, often surrounding nuclei. Monocytes and macrophages had few *mitochondria*. Mitochondria of epithelioid cells and giant cells were larger and more numerous. *Cytoplasmic filaments*, 50–60 Å in diameter, occurred singly and in bundles in all stages, but few were observed in monocytes and macrophages. Greatly increased in epithelioid cells, filaments (90–100 Å in diameter) often encircled the cytocentrum. Dense, cup-like structures (?small discharging lysosomes) occurred in the cell membrane in every stage. Contiguous epithelioid cells coalesced by fusion of apposed membranes. Regardless of length of culture (up to 6 months), epithelioid cells retained an orderly cytoplasmic organization in contrast to giant cells, which exhibited great regional variations in organelle content, innumerable small multicentric Golgi regions, and polymorphic mitochondria.—*Nat Cancer Inst Monogr* 26: 71–141, 1967.

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

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MONOCYTES UNDERGO a distinct sequence of morphological and chemical alterations, *in vivo* and *in vitro*, as they develop into macrophages, epithelioid cells, and multinucleated giant cells. Since the initial description of this transformation, practically every aspect of its cytology at the light microscope level has been extensively investigated. However, study of the fine structural aspects has lagged behind the development and use of the electron microscope in cell biology. This paper deals with the fine structural changes blood monocytes demonstrate in tissue culture. It characterizes the structure and interrelationships of various cell organelles during these changes. The intracellular events accompanying phagocytosis and changes in the cell membrane during fusion to form multinucleated giant cells are described. Additionally, observations on the fine structure of epithelioid cells and giant cells maintained in culture for prolonged periods (up to 161 days) are outlined.

HISTORICAL NOTE

As background for characterizing the ultrastructure of monocytes *in vitro*, a brief historical resumé seems appropriate. M. R. Lewis (1) first demonstrated in 1925 that, in culture, monocytes from normal blood transform into macrophages, epithelioid cells, and multinucleated giant cells. Lewis and Lewis (2) subsequently showed that the large mononuclear blood cells of all classes of vertebrates changed into ". . . macrophages similar to those found in connective tissue, liver, spleen, and lymph nodes and epithelioid cells and giant cells precisely like those found in the tuberculous lesions."

Monocyte transformation in vitro.—The cytological alterations of monocytes in tissue culture are sufficiently similar in nature and constant in occurrence to permit their being summarized (1-8). In essence, the most notable changes occurring at the light microscope level are: 1) an increase in cell size and certain cytoplasmic constituents, especially neutral red granules and mitochondria, 2) conversion of monocytes to macrophages which debride the culture of degenerating cells and cell fragments, and 3) epithelioid cell formation and coalescence to form multinucleated giant cells.

The striking similarities between these cells in culture and those in various tissues of the living organism arrested the attention of many of the early investigators of this subject (6, 8-11). For the most part, investigations centered on establishing the identity of these cells *in vitro* and *in vivo* through extensive and meticulous documentation of their cytological and functional properties.

Monocyte transformation in vivo.—Observations *in vivo* of the transformation began with M. R. Lewis' experiments on exteriorized frog lung

(5). After injection of India ink into the heart, large ink-containing monocytes lodged in lung capillaries, and after several hours they had hypertrophied and resembled macrophages identical to those observed in cell culture.

Convincing evidence for the identity and interrelationships of these cells in the living organism was obtained by Clark and Clark (12) in 1930 and Ebert and Florey (13) in 1939. In amphibian larvae, Clark and Clark (12) traced vitally stained mononuclears from the blood into connective tissue where they developed into cells morphologically identical to the indigenous population of tissue macrophages. The most unequivocal and convincing evidence establishing the transformation of blood monocytes into macrophages and epithelioid cells came from the studies of Ebert and Florey (13) with the rabbit ear chamber. They observed blood monocytes, tagged with vital new red or India ink, migrating extravascularly into connective tissue and transforming into cells morphologically and functionally indistinguishable from local tissue histiocytes. Furthermore, they noted that these cells hypertrophied and that their content of cytoplasmic granules increased. With increasing age (cells were observed up to 79 days), these cells occasionally fused to form giant cells.

Cytochemistry.—The scope of this presentation prohibits extensive review or comment on cytochemical changes accompanying the transformation of monocytes. Weiss and Fawcett (14) found monocytes of the blood devoid of stainable acid phosphatase, whereas macrophages and epithelioid cells were strongly positive. In addition to increased amounts of acid phosphatase, Cohn and Benson (15) demonstrated increased activity of two other lysosomal enzymes, β -glucuronidase and cathepsin, as well as an increase in total cell protein during transformation of mouse peritoneal monocyte-like cells into macrophages.

Reviews of various aspects of the histochemical, cytochemical, and biochemical properties of monocytes are available. Tompkins (16), Brücher (17), and Wachstein (18) present reviews of the chemistry of the monocyte. In reviewing macrophages, Jacoby (19) details the cytochemical properties of macrophages of vertebrates *in vitro*.

MATERIALS AND METHODS

Tissue culture.—The material studied consisted of Leighton tube cultures of leukocytes separated from cardiac blood of Rhode Island Red cockerels by albumin flotation (20) and grown in a medium consisting of 60% Eagle's medium and 40% chicken serum. Cultures were incubated at 37 C in an atmosphere containing 5% CO₂.

After 24 hours' incubation, the transformed monocytes were firmly attached to the glass substrate. At this time the medium was replaced. There-

after, the medium was replaced every 3 days. Before fresh medium was introduced, the pH of the old medium was measured with a Beckman pH meter. The pH ranged from 7.0–7.5, but the majority of cultures were maintained at a pH between 7.3 and 7.45.

Light microscopy.—Each culture was followed daily by light microscopy. Appropriate stages of the transformation were selected and prepared *in situ* for electron microscopy. For general cytological study, flying coverslip cultures were removed, fixed in absolute methyl alcohol, and stained with May-Grünwald-Giemsa. The remainder of the culture, growing on the floor of the tube, was used for electron microscopy.

Electron microscopy.—The cultures were prepared for electron microscopy by fixation, dehydration, and embedment in Araldite *in situ*. Portions of the embedment containing suitable cells selected by light microscopy were cut out and affixed to a small metal dowel, sectioned in a Porter-Blum microtome (M1 and MT-2) with glass knives, mounted on bare grids, and stained with either 1.0% KMnO_4 (21), $\text{Pb}(\text{OH})_2$ (22), uranyl acetate (23), or double-stained with uranyl acetate followed by $\text{Pb}(\text{OH})_2$. Sections were studied in an RCA-EMU 3F, 3G, or Siemens Elmiskop I. Methods of fixation, embedding, and mounting were developed for this investigation and have been reported elsewhere (24).

OBSERVATIONS

Light microscope.—Phagocytosis is most active between 6 and 24 hours. At 24 hours a uniform monolayer of macrophages covers the floor of the tube. Transformation to epithelioid cells begins by the 3d or 4th day and is completed by the 5th or 6th day. Giant cells are present from 9–15 days, but may appear as early as 2–4 days. The stages reported here are the original inoculum and cells cultured 1, 6, 9, 12, and 24 hours and 3, 5, 7, 11, 15, 26, 70, 80, and 160 days.

Electron microscope.—The observations on ultrastructure will be presented by stage in culture and organized under the following headings: General Cytology, Nucleus, Cytoplasm and Cell Membrane, Mitochondria, Endoplasmic Reticulum (ER), Ribosomes, Lysosomes, Vacuoles and Vesicles, Cytocentrum, and Cytoplasmic Filaments.

Circulating Monocytes (Figs. 1 and 2)

General cytology.—Circulating monocytes average 5–8 μ in diameter. The nuclear-cytoplasmic ratio is small.

Nucleus.—The nucleus shows fairly large focal chromatin aggregations (fig. 1). A small nucleolus and a few nuclear pores may be present.

Cytoplasm and cell membrane.—Endoplasm and ectoplasm are not consistently differentiated, but a small, irregular ectoplasmic zone rims the

cell. An occasional small, finger-like pseudopodium projects from the cell surface (figs. 1 and 2). Phagocytosis and pinocytosis are rarely, if ever, observed.

Mitochondria.—Mitochondria are few (2–8 in a section of a cell), and are small, oval, and orthodox in structure. Their matrix is denser than the hyaloplasm and contains no granules.

Endoplasmic reticulum.—Granular ER is present. Smooth ER is rare. Many profiles are moderately dilated by finely granular material of high electron opacity, presumably secretory material (fig. 2). Centrally, the ER runs circumferentially about the nucleus, whereas more peripherally it appears randomly organized. Circumferential distribution is the predominant pattern (fig. 1).

Ribosomes.—Ribosomes both free and attached are abundant. Polyribosomes are not observed.

Lysosomes.—Small, membrane-bounded granules containing a uniform granular material of medium-to-high electron opacity are present throughout the cytoplasm (fig. 2).

Cytocentrum.—The Golgi apparatus is small but well developed. It is roughly circular in outline and may measure 2.0–3.0 μ in diameter. Many small vesicles contain a dense secretory material, resembling that in the rough ER and cytoplasmic granules (fig. 2). The usual vesicles, vacuoles, and flattened, stacked lamellae are seen.

Cytoplasmic filaments.—Filaments are rarely observed in circulating monocytes. One or two filaments of variable length may be present.

Monocytes, 1–12 Hours (Figs. 3 Through 6)

General cytology.—Prephagocytic forms often exhibit cytological polarity (figs. 3, 4, and 6). Ergastoplasm occupies one sector, the Golgi complex another, and the nucleus, the remaining area.

All in all, the earliest changes are minimal and are manifested primarily in three organelles—nucleus, Golgi complex, and lysosomes.

Nucleus.—The nucleus moves to an eccentric location (fig. 6). Euchromatin increases while the heterochromatin banding and focal aggregations lessen (fig. 6). Nucleoli become more distinct and conspicuous (not shown).

Cytoplasm and cell membrane.—A distinct, narrow zone of ectoplasm is present (figs. 3 through 6). Pseudopodia are increased in number and length and display basal microvesicles. Dense cuplike structures with an inward convexity appear in the plasma membrane at this stage (figs. 3 through 6). They average approximately 1300 Å in diameter. Vesicles of similar dimensions containing homogeneous, electron-opaque material are situated at variable short distances from the cell membrane (figs. 4 and 5). All stages intermediate between the full complete granule in the cytoplasm and the empty concavity at the cell surface are observed. Scattered fat droplets are regularly present (figs. 3 through 5).

Mitochondria.—Mitochondria show no apparent changes.

Endoplasmic reticulum.—Granular ER is abundant (figs. 3 through 6). It may be aggregated into multiple parallel rows of flattened, elongated cisternae or occur in short sausage-shaped and vesicular forms. Smooth ER is abundant.

Ribosomes.—Free ribosomes are plentiful (figs. 5 and 6).

Lysosomes.—Lysosomes varying both in density and internal structure, and reaching even $1.0\ \mu$ or more in diameter, are more numerous and larger than in circulating monocytes (fig. 3.). They may contain myelin figures, multiple small vesicles, or punctate densities (fig. 3).

Vacuoles and vesicles.—Micropinocytotic vesicles, linearly arranged at the base of small, blunt ectoplasmic extensions, are numerous (not shown). Large vacuoles, containing material of low electron density, are numerous and appear to be pinocytotic vacuoles (figs. 3 through 6).

Cytocentrum.—The Golgi apparatus contains a triple array of vesicles, vacuoles, and flattened parallel sacs (figs. 3, 4, and 6). The Golgi internum often consists of a highly interconnected, tubovesicular network of membranous channels (fig. 6). Occasional continuities between Golgi vesicles and rough ER are noted (figs. 3, 4, and 6). Dense, membrane-bound granules (lysosomes) are increased in size and number (fig. 3). Material of similar electron opacity occurs in many Golgi vesicles and lamellae (figs. 3, 4, and 6).

Cytoplasmic filaments.—A single bundle containing 5–8 or more filaments, 50–60 A in diameter, often sweeps through the cytoplasm. Occasionally, several filaments form a loose network in the ground substance (fig. 5).

Macrophages, 6–24 Hours (Figs. 7 Through 14)

General cytology.—Macrophages exhibit a highly variable ultrastructure due primarily to the presence of numerous phagocytotic vacuoles. The total organelle content differs little from monocytes. No appreciable change is noted in any organelle with the exception of lysosomes. With phagocytosis a slight reduction of lysosomal granules occurs.

Nucleus.—Chromatin may be less margined and occurs in smaller aggregates than earlier (figs. 9 and 11). Nucleoli are evident (not shown).

Cytoplasm.—Many large phagocytic inclusions occur (figs. 8 through 11). A rim of ectoplasm is no longer present. Ectoplasm usually occurs as pseudopodial extensions (figs. 7 and 9). Fat droplets, juxtaposed to mitochondria, are common. Dense, cupped structures are not as frequently observed as previously in the cell membrane. Occasionally, one may be seen in the membrane of a phagocytotic vacuole (fig. 8).

Mitochondria, endoplasmic reticulum, and ribosomes.—These elements are not significantly changed from the previous stage.

Lysosomes.—In macrophages beginning to phagocytose, lysosomes may be increased in number; however, few lysosomes are seen in macrophages containing whole cells (presumably recently phagocytosed) or cells undergoing digestion (figs. 8, 9, and 10). Granule contents are seen extruded from the lysosome into the space between phagocytosed cells and the wall of the phagocytotic vacuole (figs. 10 and 11).

Lysosomes with membranes contiguous with the cell membrane appear in a position indicating extracellular discharge of their contents (fig. 13). Certain foci contain granules partially emptied of their contents.

Small lysosomes are particularly numerous in cells containing many phagosomes in advanced stages of digestion (fig. 11).

Vacuoles and vesicles.—Apart from phagocytotic vacuoles, large, clear vacuoles (pinocytotic vacuoles) are observed (figs. 9, 10, and 11). Phagocytosed material in all stages of digestion is present (figs. 7 through 11). Thrombocytes, the most common cell type among macrophages, are frequently phagocytosed (figs. 7 through 10). Micropinocytosis is minimal.

Cytocentrum.—The cytocentrum is larger and more highly developed than previously (figs. 10 through 12). Interconnected Golgi vesicles form a highly branched network (figs. 10 through 12). Granular material and discrete granules of high electron opacity are frequently located within the lumen of these channels of the Golgi (figs. 9 through 12). Infrequently, continuity between Golgi vesicles and rough ER is present.

Cytoplasmic filaments.—Single filaments are more numerous and often form a loose network in the cytoplasm (fig. 14). They frequently extend radially toward the cell membrane, abutting mitochondria and lysosomes. As in previous stages, they are absent in the cytocentrum.

Epithelioid Macrophages, 3 Days (Fig. 15)

General cytology.—These cells are larger than previous stages, measuring 25–35 μ in diameter. All cytoplasmic organelles are increased in number.

Nucleus.—Heterochromatin is diminished, whereas euchromatin is widely dispersed and finely granular. Single nucleoli may be twofold to threefold larger than previously.

Cytoplasm and cell membrane.—No distinct division into endoplasm and ectoplasm exists. Phagocytotic inclusions crowd the cytoplasm. Long, slender pseudopodia with small vesicles linearly arranged at their base are present. Dense, cuplike indentations are prominent and numerous in the plasma membrane. Fat droplets juxtaposed to mitochondria are numerous.

Mitochondria.—Mitochondria are slightly increased in number. Many are increased in length but show an orthodox structure. They are fre-

quently juxtaposed to cytoplasmic filaments. No branching or polymorphism is noted.

Endoplasmic reticulum.—Granular ER takes two predominant forms: 1) long, slender cisternae with flat, parallel membranes and 2) vesicles of variable diameter containing material of low electron opacity.

Lysosomes.—Lysosomes are further increased in number over earlier stages and are prominent in every part of the cytoplasm. Most common is the small, dense granule. They are more or less radially arranged within the cytocentrum.

Vacuoles and vesicles.—Phagocytotic vacuoles are present in greatest concentration in cells at this stage. They contain unrecognizable debris and whorls of membranes in myelin-figure configurations. An occasional freshly phagocytosed thrombocyte attests their continuing phagocytic ability.

Cytocentrum.—The cytocentrum is larger ($9.0\text{--}12.5\ \mu$ in diameter) than in previous stages. Flattened sacs of the Golgi element are arranged radially around the centrioles. Small, empty vesicles and lysosomal granules predominate within the Golgi internum. In propitious sections, diplosomes are present.

Epithelioid Cells, 7–160 Days (Figs. 16 Through 28)

General cytology.—Epithelioid cells are large polygonal, round, or oval cells contiguous with each other, assuming an epithelial appearance, hence the name, epithelioid. They may measure up to $40\ \mu$ in diameter. Epithelioid cells tend to maintain an essentially circular configuration. It appears that ectoplasm detaches from the cell along basally situated rows of microvesicles, restoring the cell to a roughly circular contour. Regardless of length of time in culture, these cells resemble each other sufficiently to warrant description as a single entity. Therefore, only noteworthy differences will be mentioned.

Nucleus.—The nucleus is always eccentrically situated in one pole of the cell (figs. 16, 17, 19, 20, and 24). Though usually round, it may be oval or indented by a finger-like cytoplasmic intrusion (figs. 20 and 28). Chromatin remains finely dispersed (figs. 16, 17, 19, 20, 23, 24, and 28). A variably narrow, almost inconspicuous rim of heterochromatin, occasionally minutely aggregated, may be seen as an extremely dense, highly osmophilic line (fig. 20). Nuclear pores are exceptionally numerous (figs. 24 and 25). One or more distinct nucleoli with nucleolenema are present (figs. 17 and 19).

Cytoplasm and cell membrane.—The cytoplasm contains a plethora of organelles including mitochondria, lysosomes, and large vacuoles (figs. 16, 18, 19, 20, 22, and 23). The latter, larger in diameter than any other cytoplasmic structure, are more or less uniformly distributed in the cytoplasm outside the cytocentrum. Their contents appear fluid with

uniformly suspended bits of dense material. Many of these cysts doubtlessly represent terminal stages of phagocytotic vacuoles. A distinct zone of ectoplasm, often containing abundant ribosomes and polyribosomes, rims most of these cells (fig. 20). Unequivocal phagocytotic inclusions are rare.

Cuplike structures in the plasma membrane are especially prominent (figs. 20, 24, 25, and 26). The rim and contents of these structures are similar in density to neighboring lysosomes (fig. 26). Lysosomes in direct contact with the invaginations of the plasma membrane suggest that the cupped structures represent lysosomes discharging their contents extracellularly (fig. 26).

Pseudopodia ranging in form from slender, elongated projections to bulky, irregular masses are present. Microvesicles are abundant within pseudopodia.

In early cultures (up to 26 days) many binucleated cells are present by light microscopy. In electron micrographs these are revealed as fusing epithelioid cells (figs. 24, 25, and 26). Such contiguous cells display regions where their cell membranes are in tight apposition. In places, the membranes have apparently broken down and the protoplasm of the two cells is confluent (figs. 26 and 27). Cuplike structures in the cell membrane, lysosomes, and mitochondria commonly border regions of fusion (figs. 25 and 26). Epithelioid cells may touch without fusing. Often elaborate interdigitations of the cell membrane of contiguous cells may be present (fig. 28). Similar regions suggesting coalescence of epithelioid cells are present in older cultures (70–90 days), but are not numerous. Regions of cell membrane apposition without fusion exist between epithelioid and giant cells (fig. 30).

Mitochondria.—Mitochondria are exceedingly numerous in epithelioid cells (figs. 16, 18, and 22). Many are longer than those in previous cells and may exceed $2.0\ \mu$ in length. They range from spherical and oval to rod-shaped and filamentous (figs. 22 and 23). Regardless of their length or number, polymorphic forms are rare. In 7-day cultures the outer membrane of many mitochondria is attenuated and in places discontinuous (fig. 32).

The density of the matrix varies from cell to cell and from mitochondrion to mitochondrion within the cell. Granules are seldom present within the matrix.

Endoplasmic reticulum.—Granular ER is moderately abundant and diffusely distributed throughout the cell (figs. 18, 20, 22, 23, and 24). Variable lengths ranging from short tubular profiles to elongated cisternae are present. Aggregates of stacked, parallel rows of flat elongated cisternae are occasionally encountered (fig. 23).

In certain epithelioid cells of 26 and 80 days of age, a continuous network of smooth ER channels of variable diameter radiates outwardly in all directions from the central portion of the Golgi apparatus (fig. 19).

Branching interconnections are common and focal dilations contain a finely granular material of low electron density.

Smooth-surfaced microvesicles of ER are abundant and diffusely distributed throughout the cytoplasm (figs. 16 and 23). Many are linearly arranged and may represent tangential sections of the smooth-surfaced channels outlined above.

Ribosomes.—Single RNP granules and polysomes are profusely distributed in the cytoplasm with the former predominating (figs. 18 and 22).

Lysosomes.—A conspicuously large population of lysosomal granules is the most arresting feature of epithelioid cells of all ages (figs. 16 through 24). This myriad of membrane-bound granules may be classified as follows: 1) homogeneous granules of variable density and diameter (fig. 19), 2) granules of varying diameter and density containing randomly or concentrically arranged membranes (fig. 21), 3) multivesicular bodies of variable diameter and density (fig. 22), and 4) large, irregular crystalloid inclusions (not shown). Occasionally compact phagocytotic inclusions containing recognizable cellular components may be present in 7-day epithelioid cells.

The membrane surrounding each granule is distinct. In the majority of forms its presence is accentuated by a region of lighter density immediately subjacent to the membrane, creating a submembranal halo.

Multivesicular granules increase in number in aging cells and are especially prominent in cultures of 70, 80, and 160 days (figs. 22 and 23).

Vacuoles and vesicles.—Terminal stages of phagocytotic inclusions appear to be represented by large, clear vacuoles in young epithelioid cells (5–11 days). Fresh phagocytotic inclusions are rare. Autophagic vacuoles containing degenerate mitochondria, cristae-like structures, myelin-figure membranes, or other cytoplasmic components are frequently noted (figs. 18 and 23). A third type of inclusion, which is present in all epithelioid cells and increases in frequency with age, consists of a large, irregular inclusion containing dense, amorphous areas alternating with slitlike, electron-transparent clefts (figs. 30 and 31).

Infrequently, epithelioid cells appear to be incarcerated within a membrane-bound space in giant cell cytoplasm (not shown). The viability of many such cells appears debatable since characteristics of degeneration accompany these cells: swollen mitochondria, rarefied nucleoplasm, cytoplasmic vacuolation, and degranulation.

Cytocentrum.—The cytocentrum attains its greatest size and organization in epithelioid cells. The Golgi apparatus is composed of the usual vesicles and vacuoles and flat parallel stacks of lamellae (figs. 17, 19, 20, 21, and 24). In many cells the vesicles and vacuoles of the Golgi internum form a highly interconnected tubovesicular network continuous with and composing the centralmost part of the system of smooth ER described above (figs. 19 and 21). The entire cytocentrum is pervaded by an exceed-

ingly abundant population of lysosomal granules disposed in an orderly array (figs. 17 and 19); smaller granules are situated centrally, whereas the larger ones tend to aggregate peripherally. In those cells with massive accumulations of lysosomal granules within the Golgi region, a concomitant decrease, or virtual depletion of vesicles, vacuoles, and lamellae may occur (fig. 17). In this situation the external limits and internal structure of the complex are obscured or obliterated. Occasionally, the external limits may be demarcated from the cytoplasm by a large circular bundle of filaments (*see below*).

Diplosomes are present in most cells; however, many cells in 70-day (plus) cultures appear to lack centrioles. Whether this may be attributed to an unfavorable section is a moot point.

Cytoplasmic filaments.—Cytoplasmic filaments are increased. Individual filaments measure 50–60 Å in diameter and are of indeterminate length. They occur singly or in longitudinally oriented bundles everywhere in the cytoplasm except the cytocentrum. Lysosomes and mitochondria are frequently found in the interstices of the larger bundles and between the fine network of fibers.

A distinctive annular bundle often encircles the cytocentrum, delimiting it from the remaining cytoplasm (fig. 16). The nucleus may or may not be included within its circumference. This annular bundle often branches peripherally, sweeping among cytoplasmic organelles. Often lysosomal and outer mitochondrial membranes are deformed by these bundles. Filaments within centrally situated bundles measure 90–100 Å in diameter.

Multinucleated Giant Cells, 5–160 Days (Figs. 29 Through 33)

General cytology.—Multinucleated giant cells are composed of vast syncytial expanses of cytoplasm. They may contain as many as 100, or more, nuclei. By vital microscopy, many, especially older ones, contain huge vacuoles protruding from the cell surface. Brownian movement may be observed within them. Giant cells may contain epithelioid cells within their cytoplasm. Many epithelioid cells are attached to giant cells by reciprocal interlocking of finger-like ectoplasmic processes.

The most striking ultrastructural characteristic of these large cells is the enormous regional variability in organelle concentration and structure. One area may contain only mitochondria and filaments, while an adjacent area contains predominantly membrane-bound granules.

As with epithelioid cells, giant cells are sufficiently similar to each other that a unified description may be given; therefore, only noteworthy differences will be described.

Nuclei.—Nuclei are generally round or oval and identical in structure (figs. 29 and 33). Karyoplasm is finely granular, homogeneous, and stains lightly. Heterochromatin is virtually absent. The nuclear membrane ap-

pears as an extremely dense line. Nuclear pores are numerous. Usually a single, distinct nucleolus is seen.

Cytoplasm and cell membrane.—The cytoplasm is crowded with organelles; mitochondria, filaments, and membrane-bound granules predominate. A broad zone of ectoplasm randomly interrupted by linearly disposed rows of microvesicular and pinocytotic vacuoles borders most cells. Highly characteristic, short, filiform pseudopodia arise from the cell surface. Interlocking of finger-like extensions of cytoplasm occurs between giant cells and neighboring epithelioid cells.

Mitochondria.—Mitochondria are exceedingly numerous, more so than can be accounted for by epithelioid cell fusion alone (figs. 30, 31, and 32). Polymorphism is characteristic (fig. 32). Every conceivable configuration exists: filamentous, annular, branched, angulated, etc. In an impressive number of mitochondria of 26-day giant cells, the outer mitochondrial membrane is attenuated and discontinuous in places, similar to 7-day epithelioid cells described earlier. In other mitochondria, the outer membranes are uniform and intact. Granules are not seen. Many mitochondria are juxtaposed to small groups of filaments or lipid droplets.

Endoplasmic reticulum.—Granular ER is abundant and scattered through the cytoplasm as isolated flattened channels of variable length (figs. 31 and 33).

Smooth ER predominates (figs. 30 and 33). In addition to a myriad of smooth-walled vesicles, a second form, similar to that described for epithelioid cells, is seen (fig. 33). It consists of an extensive plexiform network of variable caliber tubules, branching and anastomosing freely. In younger giant cells, this network may be radially oriented around the diplosome; however, in older giant cells (70 days plus), the arrangement appears random. Occasional foci of the cytoplasmic face are studded with RNP particles.

Aging is accompanied by an increase in the vesicular agranular ER.

Ribosomes.—Free ribosomes are plentiful (fig. 31). Aggregates of ribosomes (polysomes) are notably frequent. A distinct concentration is often found in ectoplasm.

Lysosomes.—Lysosomes are abundant (figs. 30 and 31). A distinct number of types allows categorization as follows: 1) homogeneous granules of varying diameter and density, 2) granules of varying densities with focal electron opacities, 3) multivesicular bodies of variable electron density, 4) round or irregularly shaped granules with concentric and/or electron-lucent clefts, and 5) homogeneous granules of low density with single, large electron-opaque cores. Types 3 and 4 increase with age.

Vacuoles and vesicles.—Unequivocal phagocytotic vacuoles are rare. Occasional epithelioid cells, incarcerated within vacuoles of giant cell cytoplasm, demonstrate unequivocal signs of degeneration. Whether these represent phagocytosed cells undergoing digestion or cells undergoing autolysis cannot be determined.

Micropinocytotic vesicles are more numerous in these cells than in any previous stage. They are rectilinearly arranged in rows at the base of and within pseudopodial extensions of cytoplasm.

Large, clear vacuoles (apparently pinocytotic) show random cytoplasmic distribution (fig. 29). Other enormous vacuoles, measuring several μ in diameter, contain scattered bits of unrecognizable debris.

Major portions of cytoplasm may be occupied by a myriad of uniform-sized microvesicles (fig. 29). When present, this monotonous pattern is broken only by focal areas of Golgi vesicles and large vacuoles.

Numerous electron-opaque, fat vacuoles are always present (fig. 29).

Cytocentrum.—The cytocentrum is composed of small, multicentric aggregations of characteristic vesicles, vacuoles, and parallel stacks of flattened sacs (fig. 29). These multiple aggregations are usually circumferentially distributed around nuclei, but an occasional one may be situated in the cytoplasm. Most of these small aggregations lack lysosomal granules.

Cytoplasmic filaments.—Filaments are more abundant in giant cells than in any other stage of the transformation. The close relationship to mitochondria, described in all previous cells, is present again. Broad areas of cytoplasm containing only large bundles of filaments with mitochondria in the interstices frequently occur. Filaments also occur singly as members of a loosely arranged, randomly disposed, cytoplasmic network.

DISCUSSION

The two cells most often considered to undergo transformation into macrophages are the monocyte and the lymphocyte. Much has been said and written on the subject and considerable disagreement continues to exist. Much evidence has been based on interrupted or discontinuous observation of static preparations in which the fate of individual cells could not be followed or statement of dynamic alterations be made with certainty. Only positive identification of the stem cell and continuous observation of its transformation can settle the issue conclusively.

Electron microscopy of blood monocytes in culture at 1–3 hour intervals up to 24 hours indicates they give rise to macrophages that further develop into epithelioid cells and giant cells. Monocytes show an elaborate development of the cytoplasmic organelles involved in protein synthesis, segregation, and concentration (ribosomes, rough ER, and Golgi complex). They are, therefore, cytologically equipped for transforming into macrophages. The morphological alterations occurring in culture are gradual and sequential in nature, never abrupt nor sudden. The lymphocyte does not have the cytoplasmic machinery required to explain the events observed *in vitro*. Few cells show an ultrastructure intermediate between the monocyte and lymphocyte. Were the lymphocytes alone responsible

for transforming into macrophages, a *de novo* acquisition of cytoplasmic organelles would be required. Intracellular events of this magnitude are not evident. Moreover, widespread degeneration and phagocytosis of lymphocytes indicate that the changes observed in culture more nearly coincide with the cytological properties and potentialities attributed to the monocyte.

Lewis and Lewis (2) were unable to follow any obvious direct transformation of lymphocytes, but thought cells of intermediate morphology suggested that lymphocyte transformation occurred. Carrel and Ebeling (25) found that lymphocytes died out or disappeared and that only the monocytes transformed into macrophages. Reeves (8) was unable to show lymphocytes undergoing transformation into macrophages in rat connective tissue or buffy coat of the blood. Ebert *et al.* (26) studied continuously small lymphocytes *in vivo* in the rabbit ear chamber up to 24 hours and did not observe any change in them indicative of transformation.

Small hemal lymphocytes do not attach to glass surfaces (27-29); therefore, they are either phagocytosed or discarded with a change of medium. Labeled lymphocytes, followed by autoradiography, show no evidence of transformation into macrophages (30). Inability of homogeneous populations of lymphocytes to transform into macrophages in culture has been demonstrated (16). Small hemal lymphocytes are immunologically competent cells, so their capacity to differentiate into macrophages may be restricted. It appears that most macrophages arise from monocytes, at least in cultures of normal blood leukocytes.

The mechanism responsible for morphological alterations in the monocyte remains obscure. The stimulus is present in a variety of circumstances *in vivo*, as well as in culture. Phagocytosis is not a prerequisite, since cellular hypertrophy and increase in cytoplasmic organelles (lysosomes and mitochondria) occur in homogeneous populations of monocytes where phagocytosis does not occur (15, 27). Blood monocytes migrate into extravascular loci, hypertrophy, and increase their content of neutral red-positive granules. Similar cellular modifications occur in experimental tuberculosis of the peritoneal cavity (31), as well as in a variety of other experimentally induced granulomatous diseases (32). Analogous ultrastructural changes are observed in Kupffer's cells of the liver in response to phagocytosis of particulate materials and endotoxin (33-35). Changes characteristic of the transformation occur in response to a variety of macromolecular substances, notably fibrinogen and dextran sulfate (36). The response does not appear to depend upon pinocytotic uptake of materials, since it is minimal in blood monocytes. Yet the presence in many cells of myriad small, membrane-bound granules evidences active protein synthesis before the stimulus is operative. Whatever the basic mechanism, it is clear that monocytes transform in response to a variety of different stimuli under variable environmental conditions. It appears

that the response itself represents a genetically determined one. The changes in fine structure are modulations of a basic phenotype, already established in a well-differentiated cell.

Lysosomes appear to be the major product synthesized by the cell during the transformation. Lewis and Lewis (2) showed transformation of blood monocytes *in vitro* to be characterized by an exuberant development of neutral red-positive granules in the perinuclear area and cytoplasm. It can be assumed that these positively stained bodies are lysosomes, since monocyte and macrophage granules containing hydrolytic enzymes concentrate basic dyes such as neutral red (37-39). Among the most significant features of this transformation is the appearance in large quantity of acid phosphatase (14). Since avian blood monocytes contain stainable acid phosphatase, it does not appear to be a *de novo* synthesis (40). The majority of granules in circulating monocytes measure below $0.2\ \mu$ in diameter and are therefore below the limits of resolution of the light microscope. Cohn and Benson (15) have shown increases in two other lysosomal enzymes, β -glucuronidase and cathepsin, associated with hypertrophy of monocyte-like peritoneal cells *in vitro*. Although increased amounts of hydrolytic enzymes occur following phagocytosis, circulating and prephagocytotic monocytes in culture contain detectable amounts of lysosomal enzymes. It has been postulated that phagocytosis (14) and pinocytosis (41) stimulate or initiate hydrolytic enzyme production *in vitro*. While these processes do noticeably occur, they do not appear to be the only means by which the biosynthetic activity of these cells is regulated. Circulating monocytes synthesize and store lysosomal granules in the absence of both phagocytosis and pinocytosis. Both electron-opaque material and granules (lysosomes) are present within Golgi vesicles and vacuoles of macrophages actively phagocytosing and minimally pinocytosing. Also, monocytes allowed to develop *in vitro* in the absence of phagocytotic activity actively synthesize lysosomal granules (15, 27, 42).

The fine structure of the Golgi apparatus in macrophages and epithelioid cells vividly suggests that lysosomes are formed in a manner analogous to other protein-producing cells (43, 44). Recent electron microscopic autoradiographic studies on peritoneal macrophages indicate that hydrolytic enzymes are initially synthesized in the rough ER, transferred to the Golgi apparatus, and packaged into small Golgi vesicles as primary lysosomes (45).

Discharge of lysosomal granules into phagocytotic vacuoles of blood monocytes in culture (46) resembles that described for polymorphonuclear leukocytes (47) and other macrophages (39, 48, 49). Acid-phosphatase-positive granules disappear from lung macrophages *in vitro* following phagocytosis of yeast cell walls, and acid phosphatase accumulates about the ingested product in the phagocytotic vacuole (39).

The dense, cuplike structures (1300 Å) in the cell membrane of all stages in culture most likely represent foci of lysosomal discharge. They

have not been observed in the circulating monocyte, but appear in culture. These structures fail to incorporate particulate matter (50). Furthermore, all stages intermediate between full and empty granules in the cell membrane can be assimilated to form a plausible sequence of granule discharge to the exterior. These structures appear unrelated to micropinocytosis. They appear when micropinocytosis is minimal or absent. However, structures of similar nature have been described in other cells where a relationship to micropinocytotic uptake of materials has been established (51, 52).

Additionally, lysosomes of a larger species than the small, 1300 Å diameter, ones are observed in continuity with the cell membrane, in an attitude of expelling their contents extracellularly. Granules without investing membranes have been observed adjacent to the external surface of the cell, between the membranes of juxtaposed cells, and within large pinocytotic vacuoles. These multiple expenditures of lysosomes explain their reduction to almost complete depletion in macrophages actively phagocytosing. The role of lysosomes in digestion of material phagocytosed by cells is well known and needs no elaboration in this context.

Whether the extracellular discharge of lysosomes is of physiological significance or related to the tissue culture environment is not known. In tissue cultures, degenerating cells are seen adjacent to macrophages with exteriorized granules. Release of lysosomes extracellularly by the monocyte and derivative cells may initiate or hasten degradation of material in advance of, or in preparation for, phagocytosis. Extracellular discharge of lysosomes *in vivo* might facilitate movements of this cell through body tissues by regulating the consistency of extracellular components of connective tissue. Small lysosomes almost always occur in the vicinity of membranes in the process of fusion. The activity of certain lysosomal enzymes may be required to mediate membrane changes associated with cell fusion. Discharging lysosomes extracellularly, representing a merocrine-type of secretion, appears to be a normal function of monocytes.

In epithelioid cell fusion to form giant cells, another mechanism may be involved. Contiguous epithelioid cells often display elaborate ectoplasmic interdigitations. These may lead to fusion by periodic breaks in the apposed membranes, followed by fusion of these membrane segments to form rows of vesicles with communicating strips of cytoplasm intervening between them. Policard *et al.* (53) and Gusek (32) have observed a similar interlocking in epithelioid cells *in vivo*, and postulate a functional role in giant cell formation.

Cytoplasmic filaments in monocytes and their progeny in tissue culture are similar to those found in guinea pig and rabbit peritoneal macrophages and human blood monocytes (54). Similar filaments have been observed in normal and leukemic leukocytes, in healthy and diseased

cells of the hematopoietic and reticuloendothelial systems (55, 56), and in fibroblasts of healing wounds and tissue culture (57). Additionally, association of similar filaments with a dynamic role has been made in slime molds (58) and ascidians (59). Bessis and Breton-Gorius (60) have recorded cinematographically multiple lobulations of nuclei in blood monocytes and lymphocytes in response to oxalate anticoagulants. Associated with these configurational changes are filaments of similar diameter to those in cells described above. Epithelioid cells in culture usually have round or oval nuclei. Perhaps it is more than mere coincidence that in epithelioid cells with indented or polymorphic nuclei, bundles of filaments are more frequently found in association with the nuclear indentations.

In cultured monocytes a relationship between cell shape and movements and cytoplasmic filaments has been observed. Quiescent epithelioid cells can be manipulated to change from a rounded or polygonal form to a spindle shape, or fibroblastic form, and back again. Associated with these movements is a change in the number and distribution of filaments. Spindle-shaped cells have an increased number of filaments, singly and in bundles, all oriented in the direction of the long axis of the cell! Fewer filaments, randomly distributed, are found in polygonal cells before and after the change to the fibroblastic form. Additionally, their presence as an annular bundle encircling the cytocentrum in epithelioid cells and around phagocytotic vacuoles in macrophages signifies a role in maintaining the shape and organization of the cytoplasm. Their frequent association with ectoplasm suggests a dynamic role in undulation of the cell membrane.

Individual filaments in peripheral cytoplasm measure 50–60 Å in diameter, whereas those associated with the central bundle, encircling the Golgi complex, measure 90–100 Å in diameter. Structural differences between the two are not obvious. Differences in diameter may therefore reflect different physical states or two separate species of filaments.

A close topographical relationship to mitochondria is always pronounced. Many mitochondria are longitudinally juxtaposed to large bundles of filaments. Evidence has been obtained in *Physarum polycephalum* that similar cytoplasmic filaments are capable of splitting ATP and are contractile in nature (58, 61). Intimate association in monocytes may signify a functional dependency of the filaments upon mitochondria. Such close proximity would tend to assure a continuous supply of energy for contraction.

Foci of probable attachment to cell membrane, nuclear membrane, and membranes of lysosomes and vacuoles are observed and may in some way be related to translocation of these organelles and inclusions to specific locations within the cytoplasm (54).

Increases in the concentration of filaments, with increasing time in culture, may reflect a phenomenon observed by Biberfeld (62) in mono-

layer cultures of parenchymal rat liver cells. Accumulation of filaments was correlated with length of time *in vitro*. It was postulated that the increase was due to a change in the functional and dynamic state of the cell, induced by transfer to the tissue culture environment.

The ultrastructure of monocytes, macrophages, and epithelioid and giant cells in tissue culture is similar to that of mononuclear phagocytes ubiquitously distributed throughout various tissues of the living organism (monocytes and macrophages: 24, 31-33, 38, 45, 46, 48, 49, 51, 53, 54-57, 60, 63-77; epithelioid cells: 31, 32, 53, 78; giant cells: 31, 32). Enough information is becoming available on monocytes and derivative cells to adequately justify this conclusion. There is little question that more similarities than differences exist.

A more meaningful interpretation can be derived from the ultrastructural diversity observed in monocytes in tissues culture when these cells are examined in light of their functional potentialities. The functional quintessence of monocyte development *in vitro* appears to be an enhancement or aggrandizement of the biosynthetic machinery of the cell, expressed in an exceedingly abundant synthesis and storage of lysosomal granules.

Considering the ultrastructural and biochemical data available, tentative conclusions on the function of each cell stage may be drawn. The blood monocyte serves as the stem cell, the counterpart in the blood of the tissue histiocyte or macrophage. One of its primary functions appears to be the augmentation of its cytoplasmic apparatus for synthesis and storage of lysosomal granules, for situations requiring phagocytosis. It is a well-differentiated cell possessing all the necessary organelles required for synthesis and secretion. There is no question that the macrophage functions in phagocytosis and enzymatic degradation of effete cells, tissue debris, microbial organisms, and macromolecular catabolic products derived from metabolic activities of the organism.

The epithelioid cell is a macrophage. It represents a stage of intensified secretory activity associated with the formation and accumulation of an overabundance of lysosomal granules. In addition to its enhanced biosynthetic capacity, it exhibits an enhanced phagocytotic capacity which in all probability is based upon its rich lysosomal content. It represents the apex of monocyte organization and possesses all the earmarks of a macrophage. The functional capabilities and ultrastructural relatedness of these three cells, the monocyte, macrophage, and epithelioid cell, suggest that they are "merely different functional forms of the same cell" (2).

The giant cell remains an enigma. Its ultrastructural appearance defies its classification as a defunct or debilitated cell. Rather, its abundance of cytoplasmic organelles and durability suggest presently unknown func-

tions for this cell in the tissues of the body. It differs immensely in structural organization from cells which fused to form it. Massive accumulations of lysosomes evidence continuing protein synthesis. Massive concentrations of mitochondria, highly active cell membrane, large quantities of pinocytotic vacuoles, and micropinocytotic vesicles indicate cytoplasm engaged in active transport, not unlike other giant cells, such as the osteoclast (79-81). Its ability to sequester inert foreign bodies is well known. Additional metabolic activities of such a cell with capabilities of phagocytosis, active transport, and sequestration remain obscure and must await future investigation.

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Key to Abbreviations

AV	Autophagic vacuole	M	Monocyte
C	Cuplike structure	Ma	Macrophage
Ce	Centriole	MVB	Multivesicular body
Cr	Chromatin	My	Myelin figure
Cm	Cell membrane	Nu	Nucleolus
Ec	Ectoplasm	N	Nucleus
Ect In	Ectoplasmic interdigitation	Om	Outer mitochondrial membrane
Ep	Epithelioid cell	Ps	Pseudopodium
Fl	Filament	P, NP	Pore or nuclear pore
Flb	Bundle of filaments	PhV	Phagocytotic vacuole
G	Golgi region	R	Polyribosomes
Gc	Giant cell	RNP	Ribonucleoprotein, ribosome (free)
Ger	Granular endoplasmic reticulum	Ser	Smooth-surfaced endoplasmic reticulum
Gr	Granule	Th	Thrombocyte
GVe	Golgi vesicles	V	Vacuole
I	Inclusion	Ve	Vesicle
L	Lipid		
Ly	Lysosome		

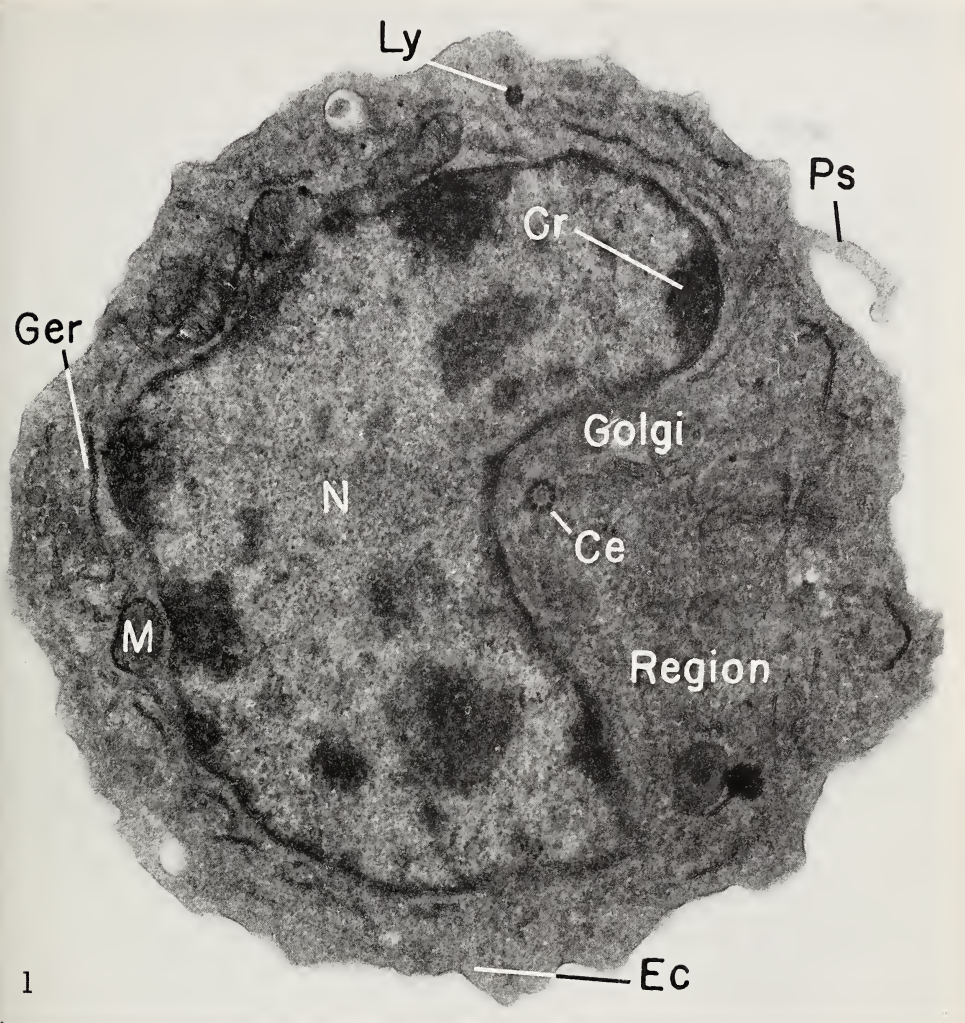


FIGURE 1.—Circulating monocyte. This cell contains a typical crescent-shaped nucleus (N), with large, peripheral focal aggregations of chromatin (Cr). The nuclear hof encloses a large, well-developed Golgi region, containing a centriole (Ce) within. A few lysosomal granules (Ly) of variable electron opacity are widely scattered in the cytoplasm. Several mitochondria (M) are present among circumferentially oriented flat channels of rough endoplasmic reticulum (Ger). A narrow band of ectoplasm (Ec) rims the cell and extends into a single, short filiform pseudopodium (Ps.) Glutaraldehyde post OsO_4 fixation; lead citrate stain. $\times 25,450$

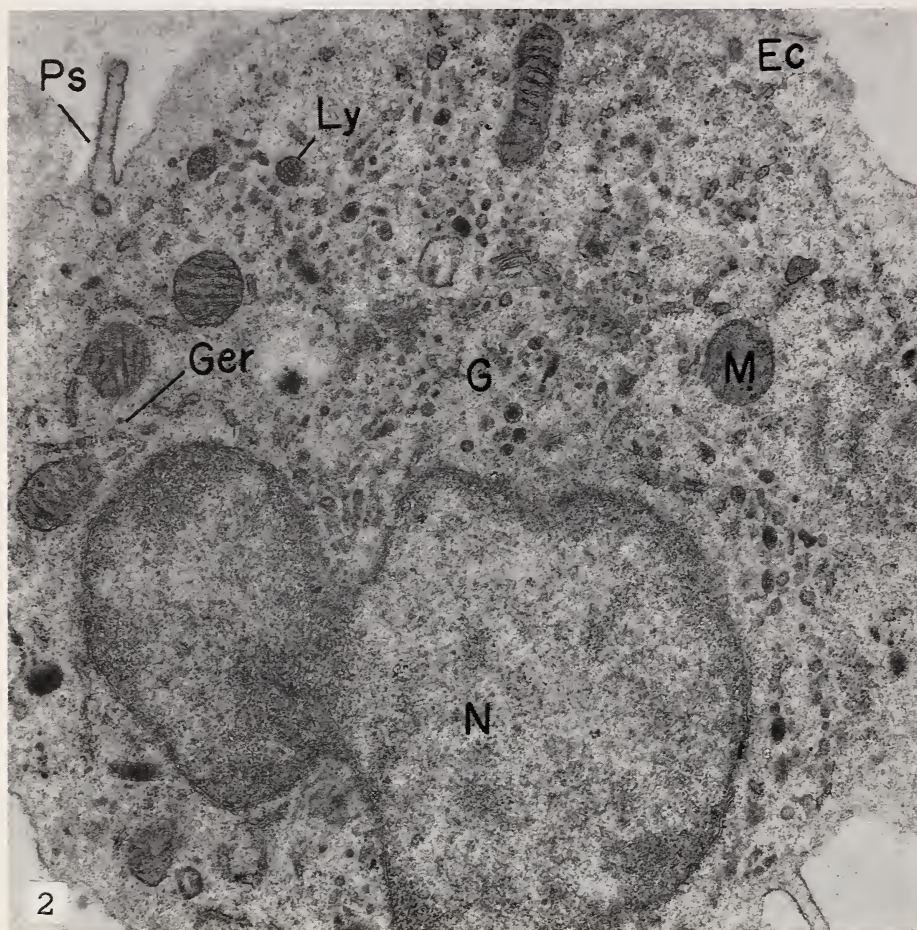


FIGURE 2.—Circulating monocyte. This cell contains a myriad of small, dense, lysosomal granules (Ly) in the cytoplasm and Golgi region. The Golgi region (G) is small but well organized. Many cisternae of rough endoplasmic reticulum (Ger) contain electron-opaque secretory material. OsO_4 fixation; KMnO_4 stain. $\times 24,775$

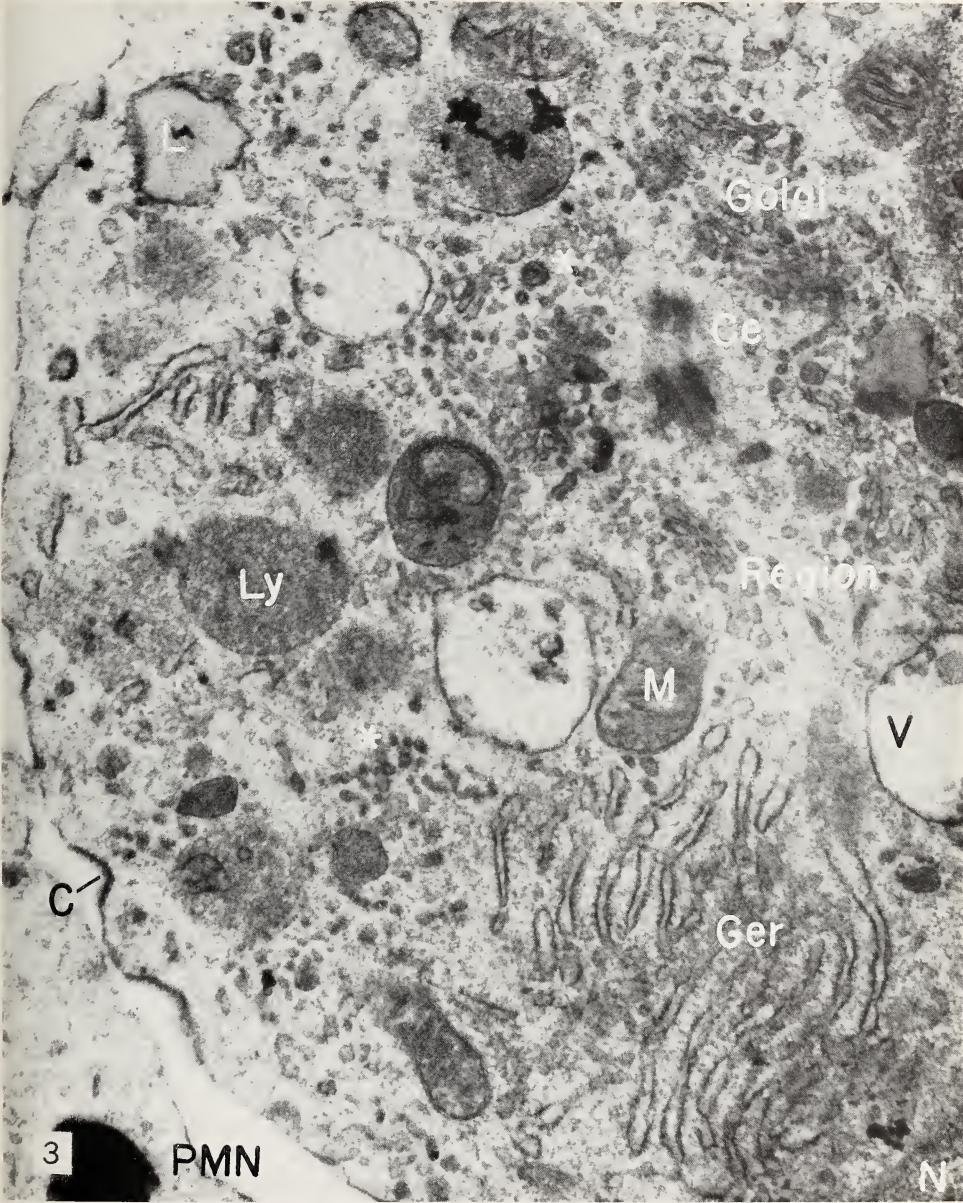


FIGURE 3.—Monocyte, 6-hour culture. Several enlarged lysosomes (Ly) are in the margin of the Golgi region and adjacent cytoplasm. Several large pinocytotic vacuoles (V) surround the Golgi externum. Channels of rough endoplasmic reticulum (Ger) are dilated by electron-lucent material. OsO₄ fixation; KMnO₄ stain. $\times 30,250$

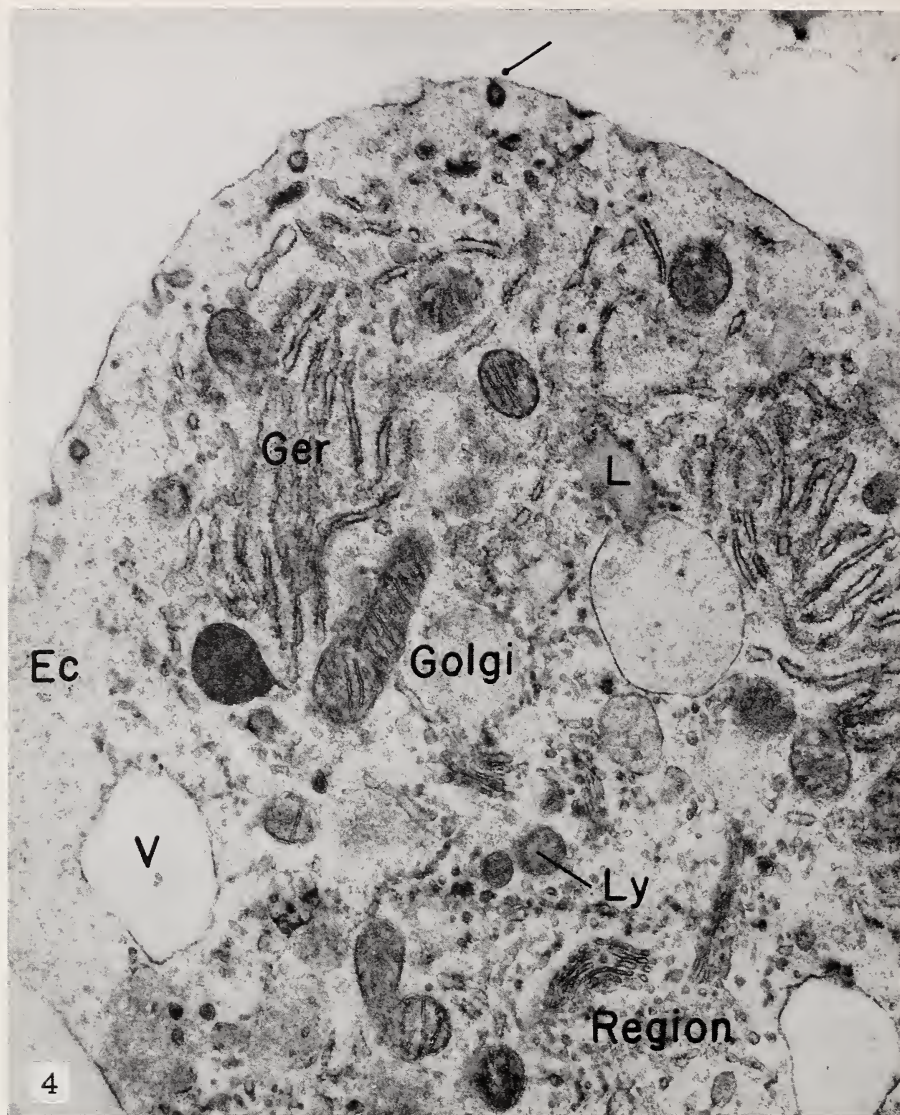


FIGURE 4.—Monocyte, 6-hour culture. The cell membrane contains several electron-opaque vesicles. One is seen touching an invagination of the cell membrane (*arrow*). Dense material within channels of rough endoplasmic reticulum (Ger) nearby is similar to that within the vesicles. Abundant rough ER (Ger) and a large Golgi region containing electron-opaque granules (Ly) are present. OsO_4 fixation; KMnO_4 stain. $\times 21,700$



FIGURE 5.—Monocyte, 6-hour culture. Multiple dense, cup-shaped structures, averaging 1200 Å in diameter, are seen near to, or contiguous with, the cell membrane. One (arrow) appears to be extruding its contents. A loose meshwork of filaments (FI) and many free RNP granules (*within dashed oval*) are present. Granular endoplasmic reticulum (Ger) is abundant and a few channels contain dense material (*). OsO₄ fixation; KMnO₄ stain. $\times 24,200$

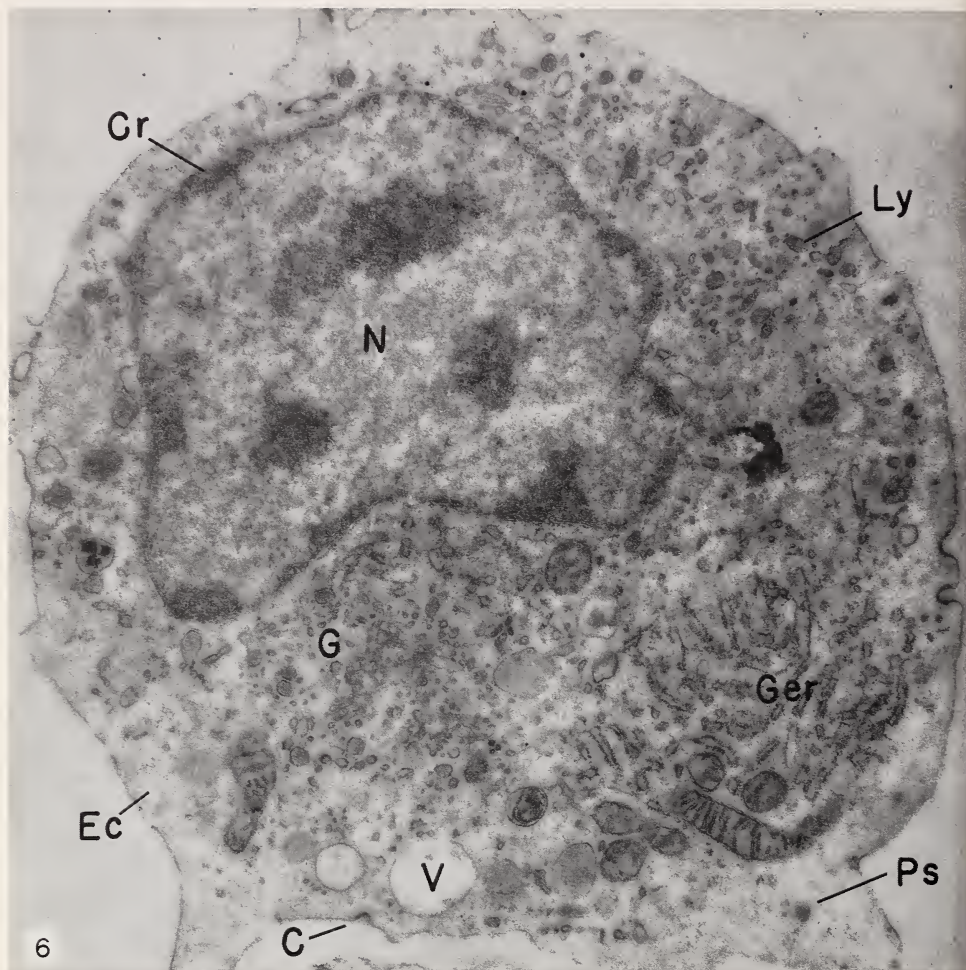


PLATE 8

FIGURE 6.—Monocyte, 9-hour culture. This prephagocytic monocyte demonstrates cytological polarity (*cf.* figs. 3 and 5) with nucleus, Golgi region, and rough endoplasmic reticulum occupying different sectors. Nuclear chromatin is less margined and aggregations are smaller. Small lysosomal granules (Ly) are numerous. Many channels of rough endoplasmic reticulum are dilated by electron-opaque material, as are many vesicles and cisternae of the Golgi apparatus. Plexiform arrangement of the Golgi is conspicuous. Long pseudopodia (Ps) extend from the cell. Cup-shaped structure (C) measures approximately 950 Å in diameter. OsO₄ fixation; Pb(OH)₂ stain. × 26,625

FIGURE 7.—Macrophage, 24-hour culture. A thrombocyte (Th) is encompassed by pseudopodial extensions (Ps₁) of ectoplasm (Ec). A pseudopodium (Ps₂) from another cell encircles a neighboring thrombocyte. The cytoplasm contains a myelin figure (My) and several lysosomes (Ly). OsO₄ fixation; KMnO₄ stain. × 18,000

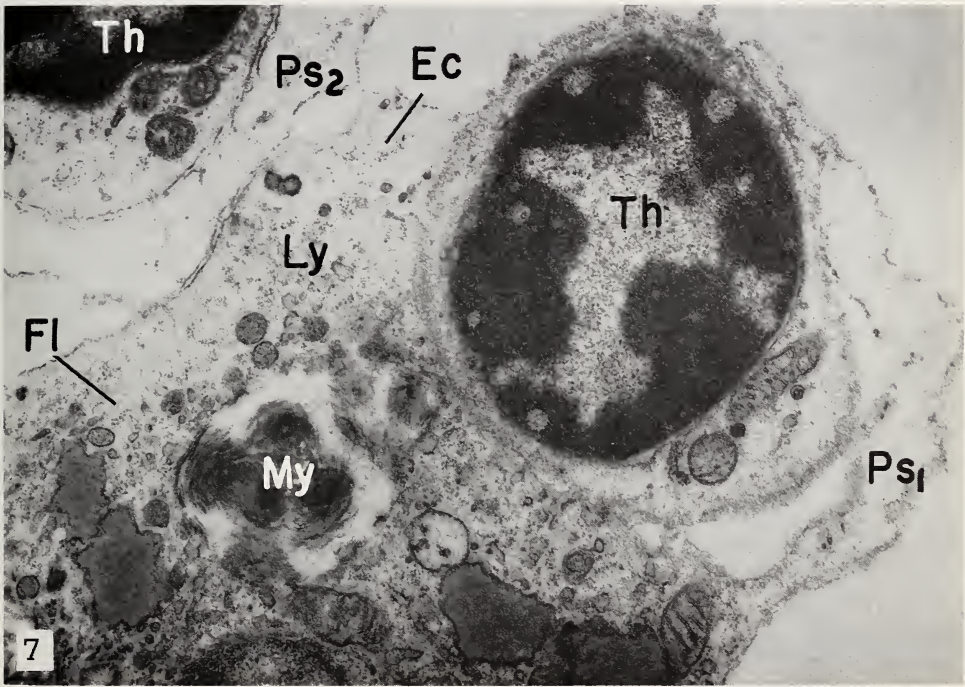


PLATE 9

FIGURE 8.—Macrophage, 24-hour culture. A phagocytotic vacuole (PhV) contains a cell undergoing dissolution. A recently phagocytosed thrombocyte (Th) lies within a phagocytotic vacuole containing a small, cuplike structure (C) in its membrane. OsO₄ fixation; KMnO₄ stain. $\times 16,000$

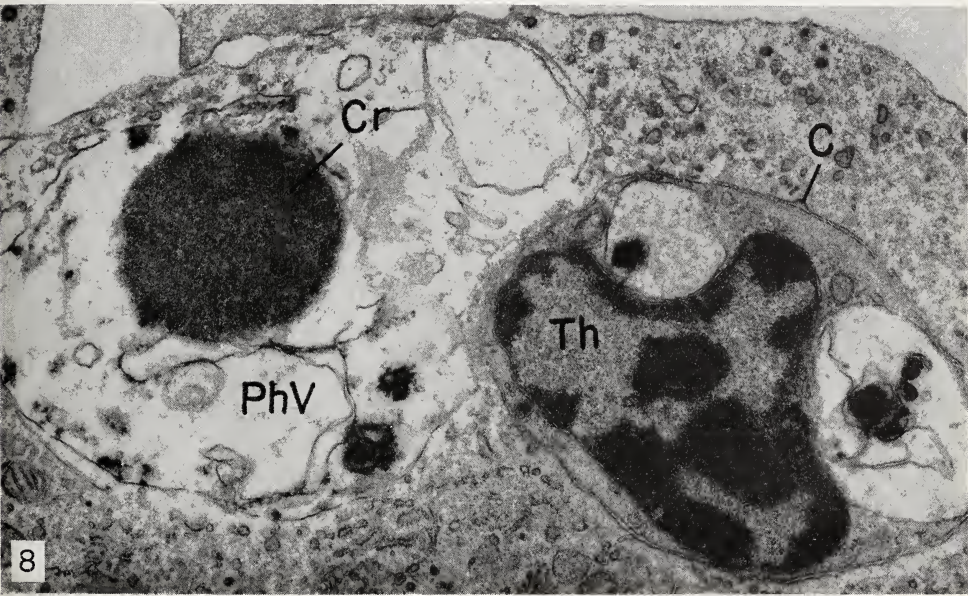


PLATE 10

FIGURE 9.—Macrophage, 9-hour culture. Large phagocytotic vacuole (PhV) and small phagocytotic inclusions bordering the Golgi region (G) attest previous phagocytotic activity in this macrophage. Rough endoplasmic reticulum (Ger) and free ribosomes (R) abound in the ectoplasm. Pseudopodial extensions (Ps_1 and Ps_2) arising from ectoplasm peripheral to the nucleus surround a thrombocyte (Th_1). *Note* nuclear shape, conforming to the outlines of forming PhV. Nucleus (N) shows only thin rim of marginal chromatin, without focal aggregations. Many Golgi vesicles contain electron-opaque material (*). Cell in *upper left corner* is a typical thrombocyte (Th_2). OsO_4 fixation; $Pb(OH)_2$ stain. $\times 25,450$

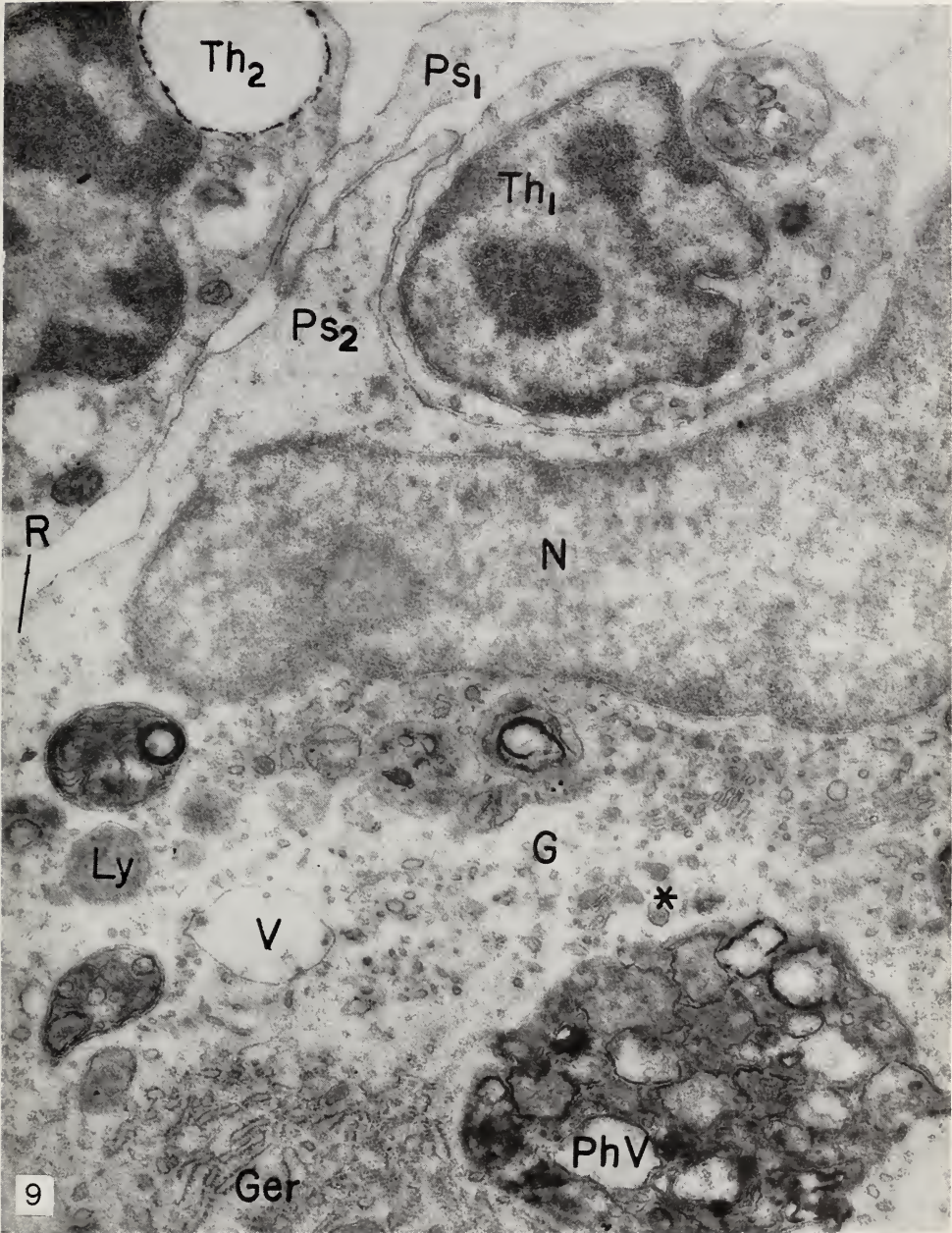


PLATE 11

FIGURE 10.—Macrophage, 12-hour culture. A recently phagocytosed thrombocyte (Th) is completely enclosed within a phagocytotic vacuole. Membranes of two lysosomes (LY_1 and LY_2) are fused with the membrane of the phagocytotic vacuole. An extremely electron-dense substance (doubtless lysosomal material) is seen between the intact cell membrane of the phagocytotic vacuole (*arrows*), and is directly continuous with the core of the lysosomes. *Note* smaller satellite lysosomes around LY_1 . OsO_4 fixation; $KMnO_4$ stain. $\times 34,200$

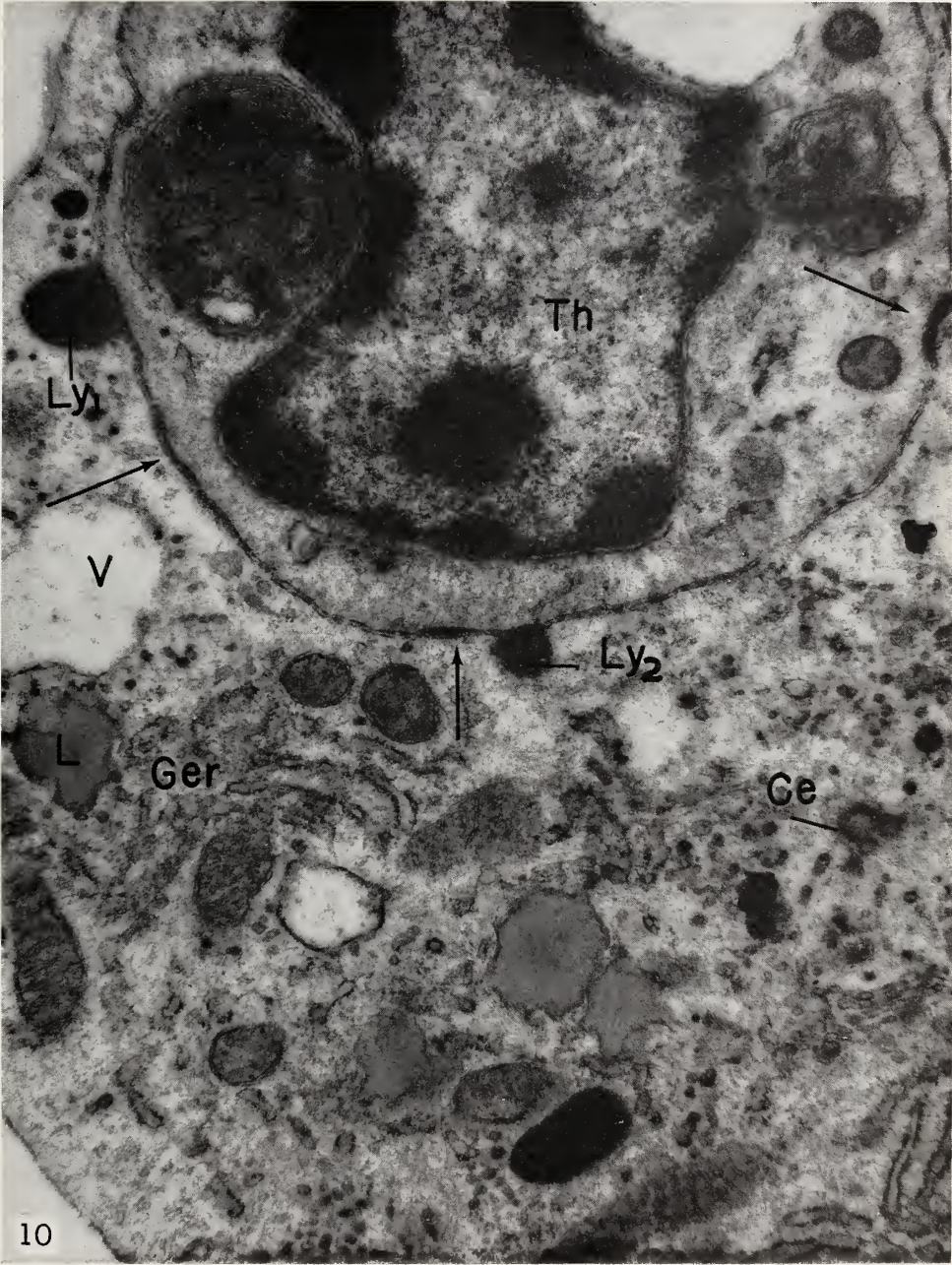
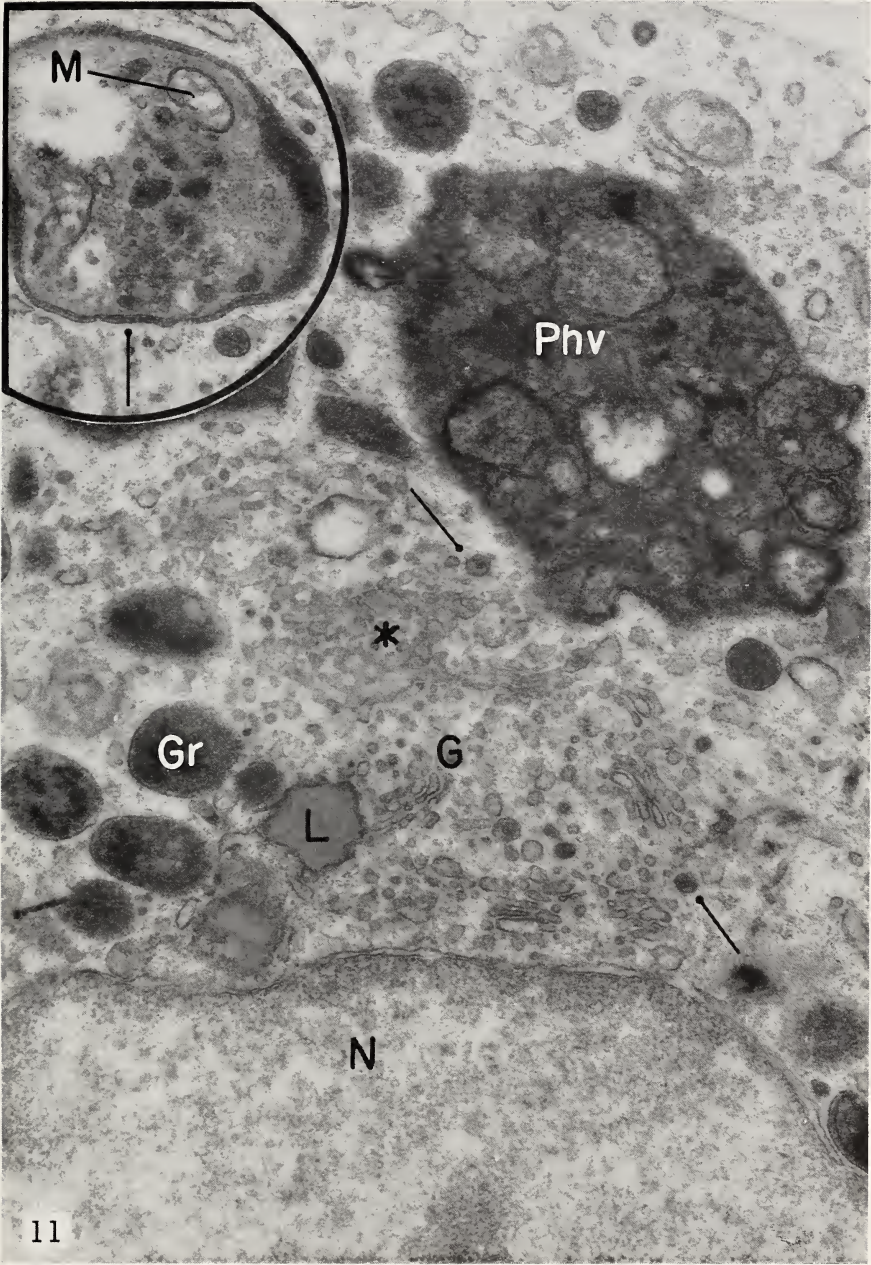


PLATE 12

FIGURE 11.—Macrophage, 24-hour culture. A phagocytosed cell (PhV) showing advanced digestion is seen in close proximity to the Golgi region (G). The large, membrane-bound granules (Gr) within the Golgi region and adjacent cytoplasm probably represent smaller phagosomes. Continuities between cisternae of the Golgi are evident (*). Several vesicles contain electron-dense material (*arrows*). *Inset* shows a dense band of material (*arrow*), probably of lysosomal origin, surrounding a phagocytosed cell containing recognizable organelles (M). OsO₄ fixation; KMnO₄ stain. $\times 41,000$; *inset* $\times 41,000$



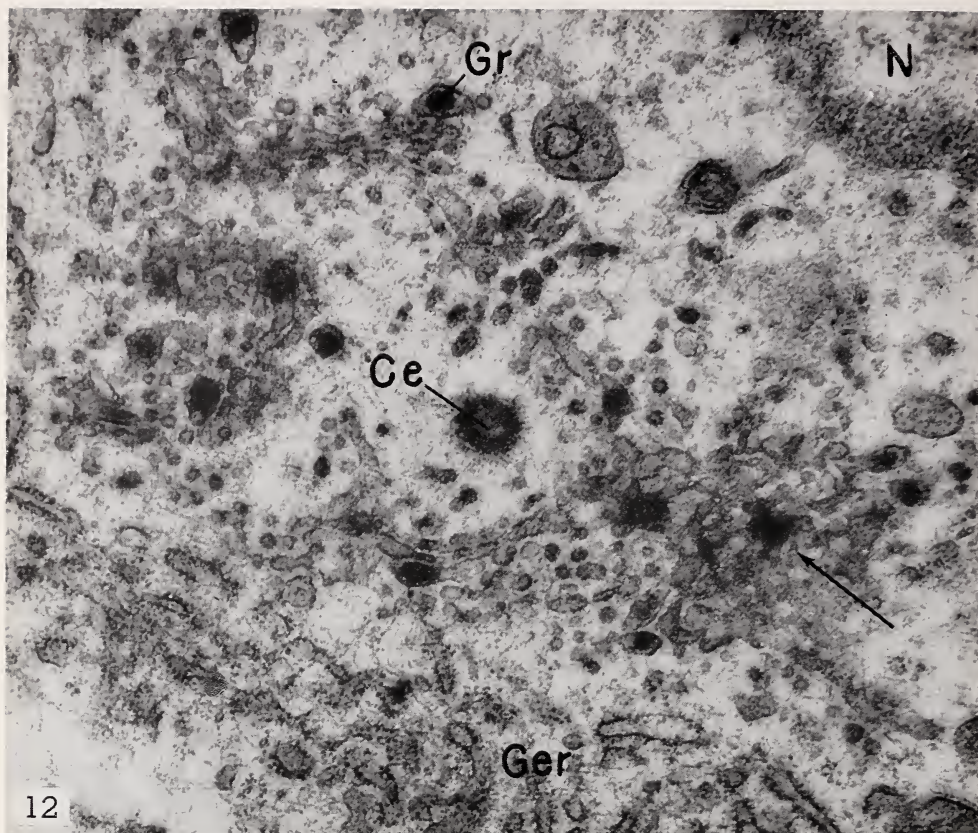


FIGURE 12.—Macrophage, 12-hour culture. This micrograph demonstrates the Golgi complex. Continuity between Golgi vesicles and lamellae is marked. Electron-dense granules (Gr) and material (*arrow*) resemble cytoplasmic granules. OsO₄ fixation; KMnO₄ stain. $\times 47,400$

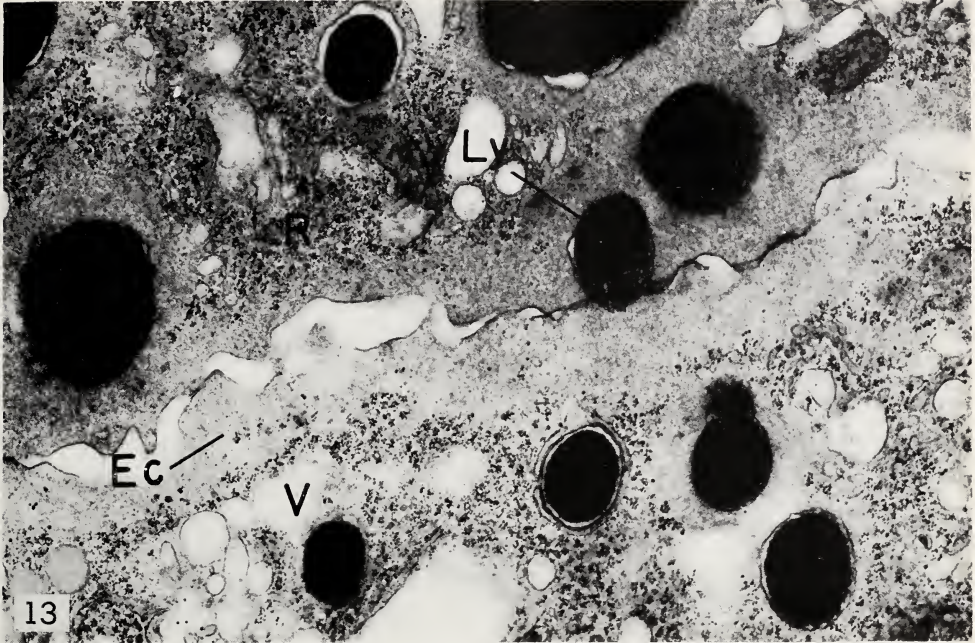
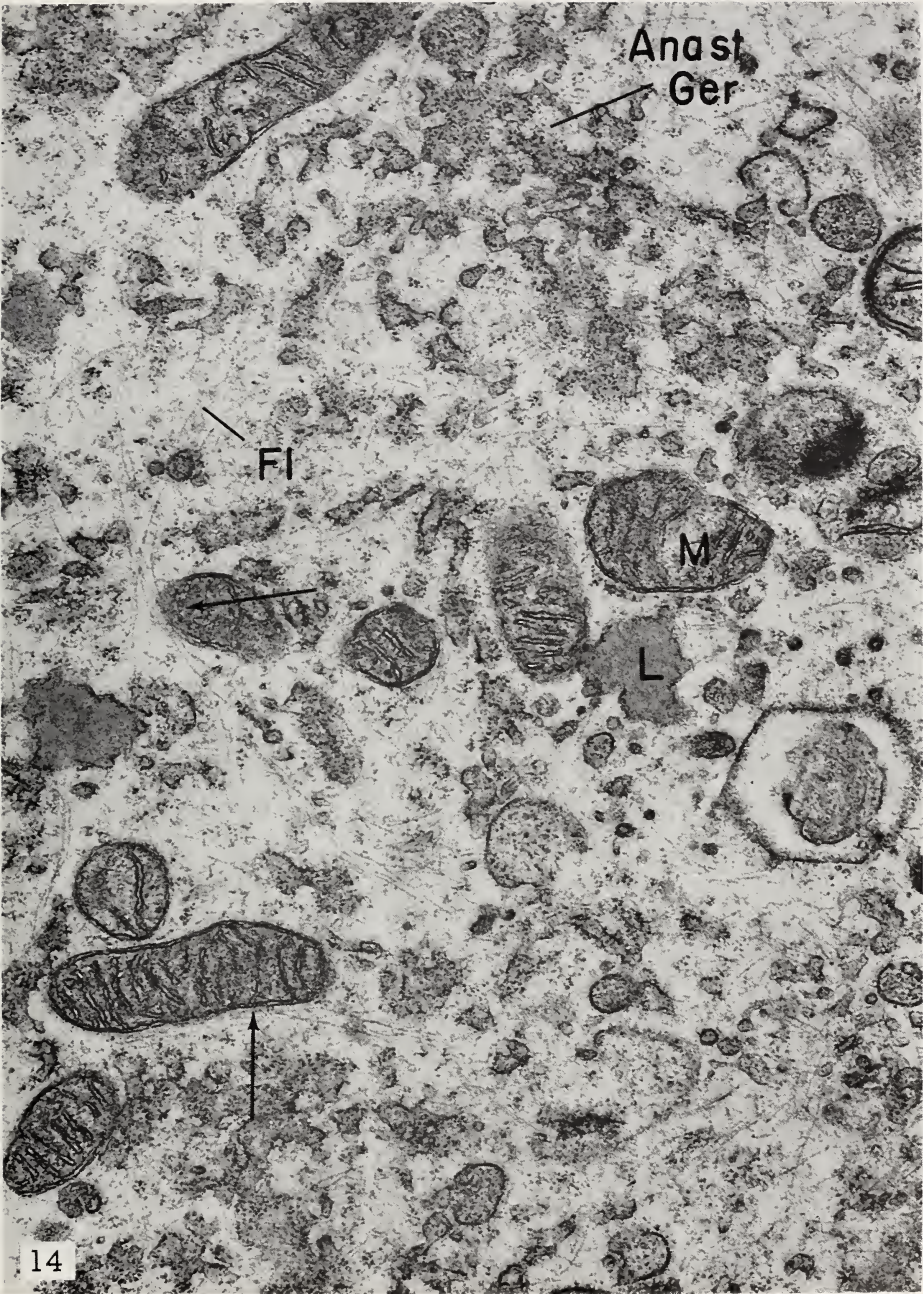


FIGURE 13.—Macrophage, 24-hour culture. A large lysosome (Ly), approximately $0.4\ \mu$ in diameter, appears to be emptying its contents at the cell surface into the space between two macrophages. Note ectoplasmic border (Ec) and high concentration of ribosomes (R) and pinocytotic vacuoles (V) in both cells. Glutaraldehyde post OsO_4 fixation; $\text{Pb}(\text{OH})_2$ stain. $\times 23,200$

PLATE 15

FIGURE 14.—Macrophage, 24-hour culture. Plexiform arrangement of the granular endoplasmic reticulum is particularly evident (Anast Ger). A loose meshwork of filaments (F1) pervades the cytoplasm. A close topographical relationship with mitochondria is noted (*arrows*), OsO_4 fixation; KMnO_4 stain. $\times 29,225$



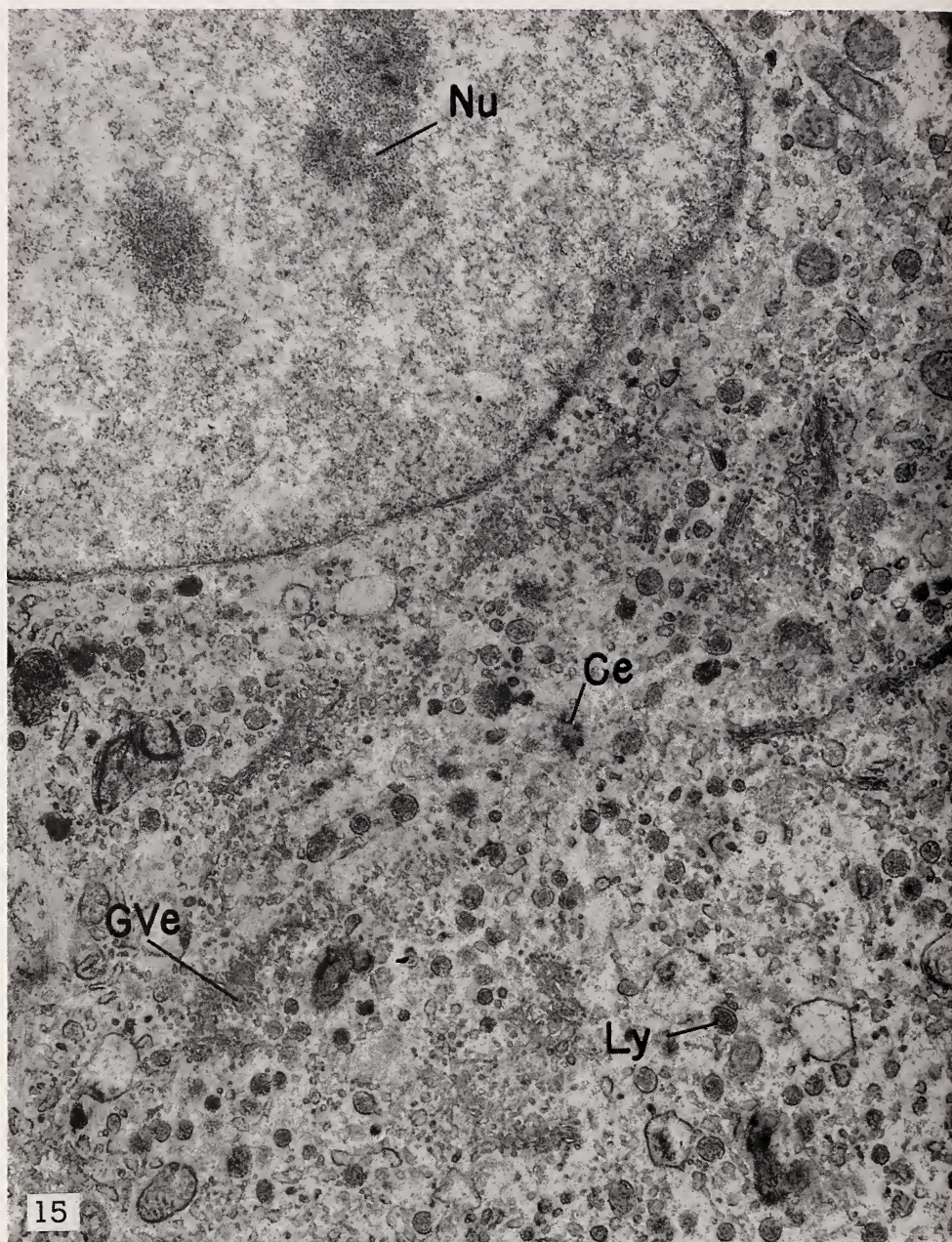


FIGURE 15.—Epithelioid cell, 3-day culture. The Golgi lamellae and vesicles (GVe) are arranged in a radial pattern around the centriole (Ce). *Note* the multiple small lysosomal granules (Ly) within the Golgi region. OsO_4 fixation; KMnO_4 stain. $\times 16,000$

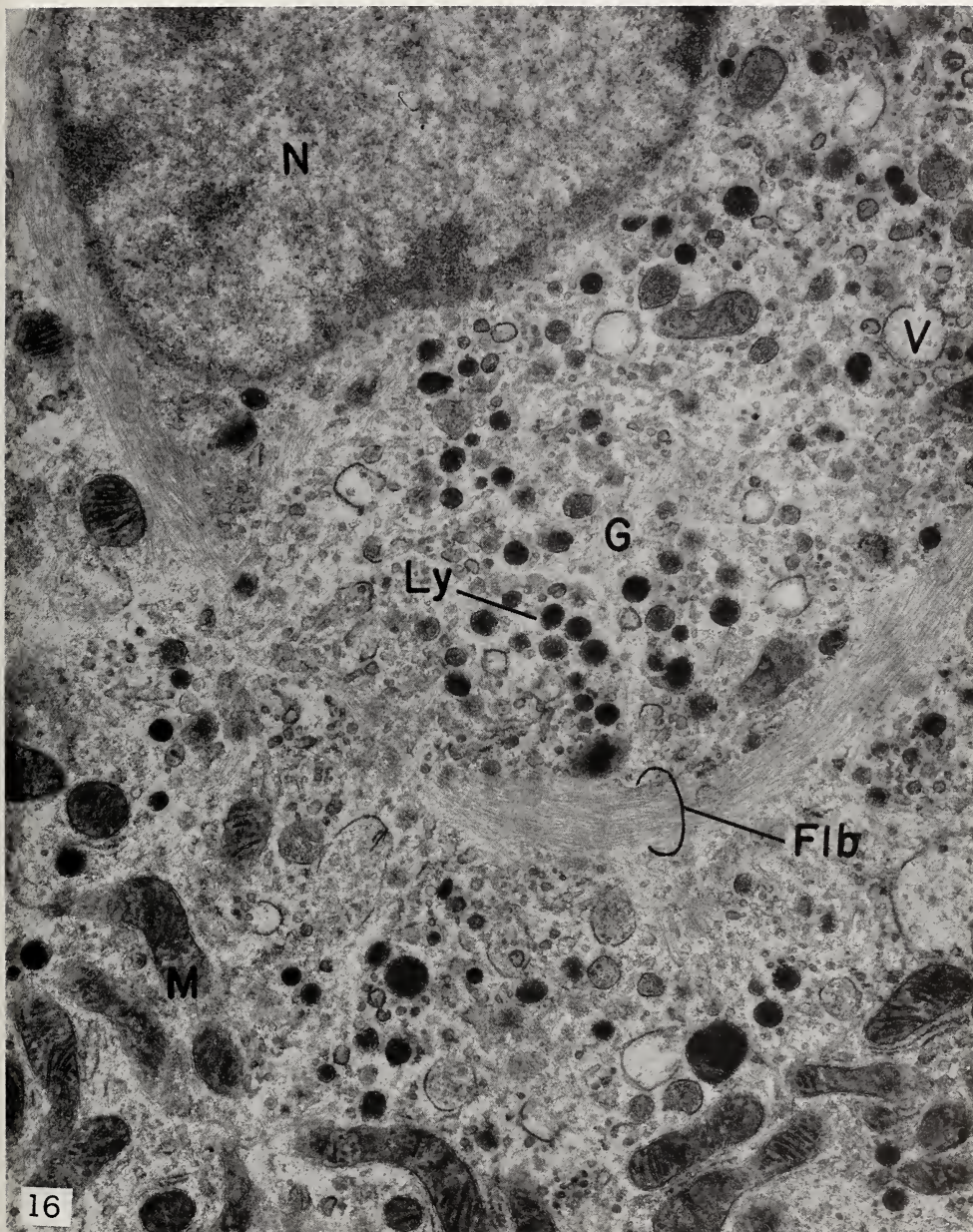


FIGURE 16.—Epithelioid cell, 7-day culture. This large bundle of filaments (Flb) encircles the nucleus (N) and Golgi region (G). Mitochondria (M) and lysosomes (Ly) are numerous. OsO_4 fixation; KMnO_4 stain. $\times 18,400$

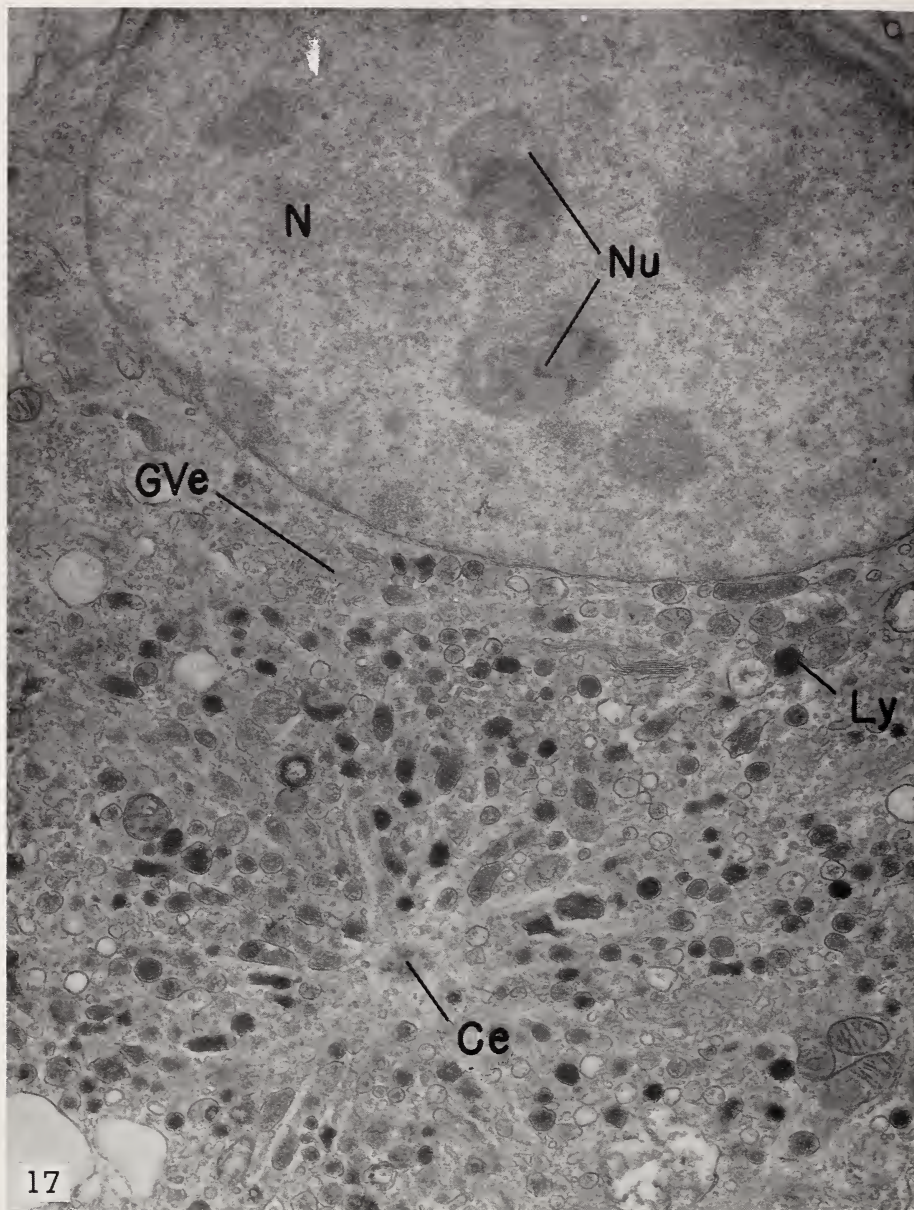


FIGURE 17.—Epithelioid cell, 15-day culture. The cytocentrum is conspicuous due to radial arrangement of lysosomes (Ly). Note that much of the dense material is located within branched Golgi lamellae (*cf.* fig. 12). The nucleus (N) contains a prominent nucleolus (Nu), OsO_4 fixation; KMnO_4 stain. $\times 13,400$

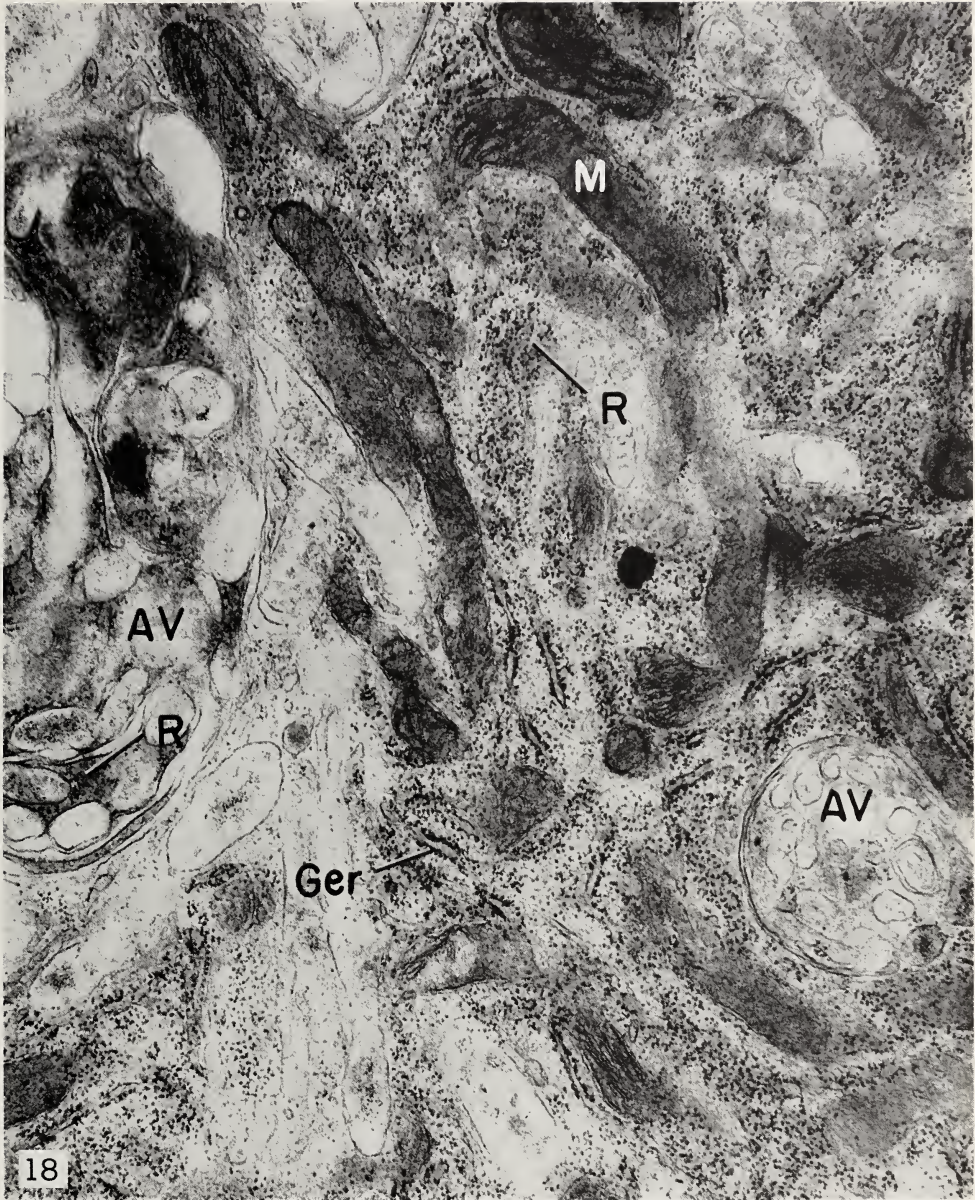


FIGURE 18.—Epithelioid cell, 15-day culture. This portion of cytoplasm is especially rich in granular endoplasmic reticulum (Ger), RNP (R), and mitochondria (M). Autophagic vacuoles (AV) contain membranes, vesicles, and RNP particles. Glutaraldehyde post OsO_4 fixation; $\text{Pb}(\text{OH})_2$ stain. $\times 32,700$

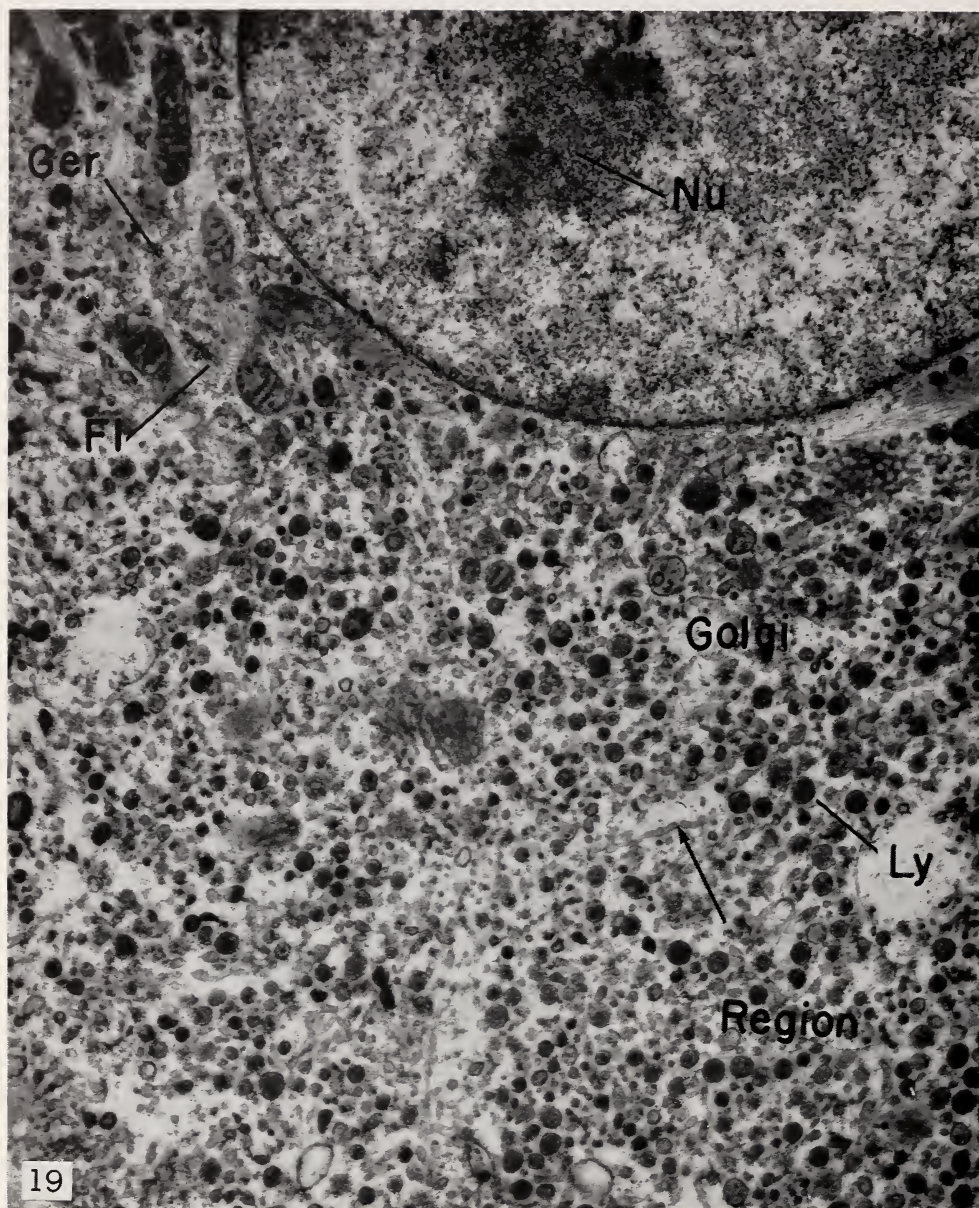


FIGURE 19.—Epithelioid cell, 26-day culture. Myriad small lysosomal granules (Ly) are present within this cell's enlarged Golgi region. Note tubular channels (*arrow*). Juxtannuclear filaments (Fl) are present. OsO₄ fixation; Pb(OH)₂ stain. $\times 17,000$

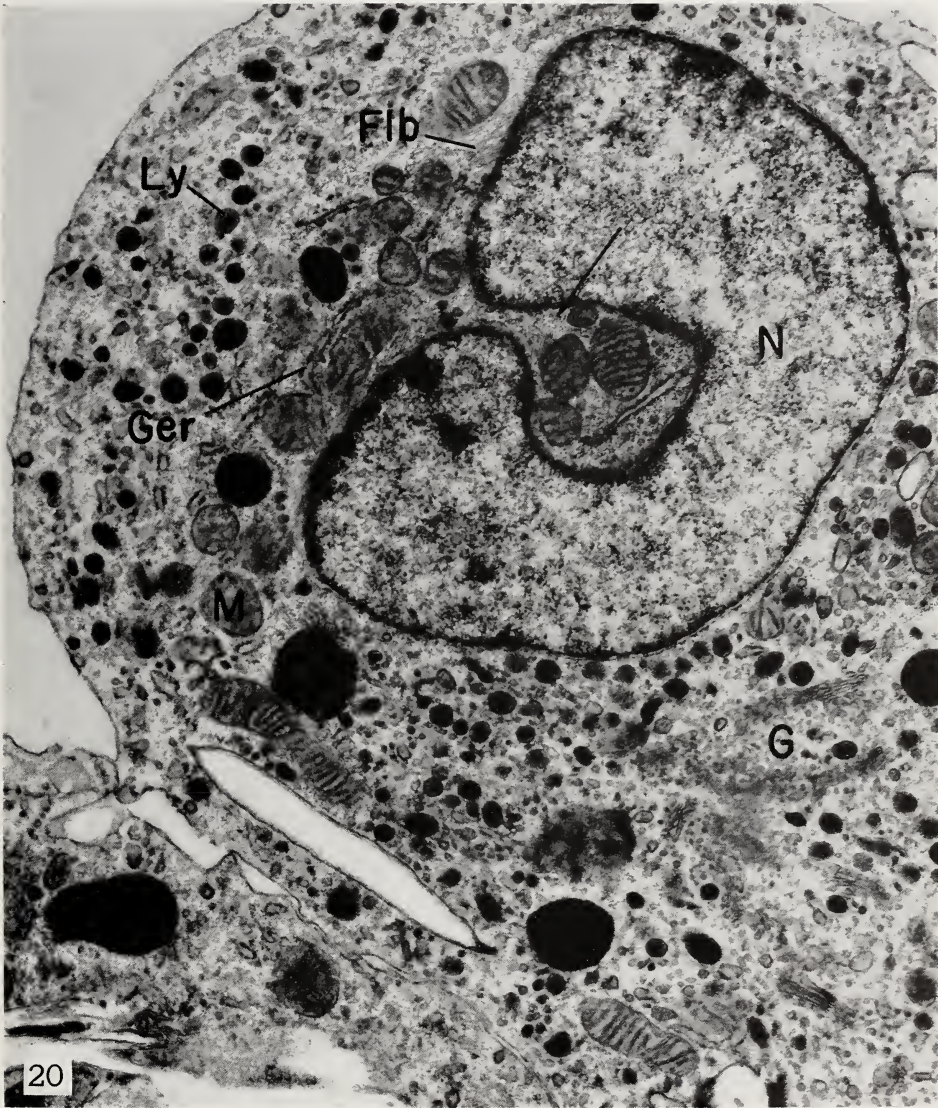


FIGURE 20.—Epithelioid cell, 26-day culture. Several bundles of filaments (Fib and arrow) are present in and near the nuclear indentation. Note rich population of lysosomes (Ly) and mitochondria (M). Granular endoplasmic reticulum (Ger) is fairly abundant. OsO_4 fixation; $\text{Pb}(\text{OH})_2$ stain. $\times 22,550$

PLATE 22

FIGURE 21.—Epithelioid cell, 80-day culture. The Golgi region in this cell is set off from the cytoplasm by an encircling bundle of filaments (Flb). Interconnections between Golgi lamellae are prominent (*). *Note* large vacuoles and variegated nature of granules (Ly), OsO₄ fixation; uranyl acetate and Pb(OH)₂ stain. $\times 24,475$

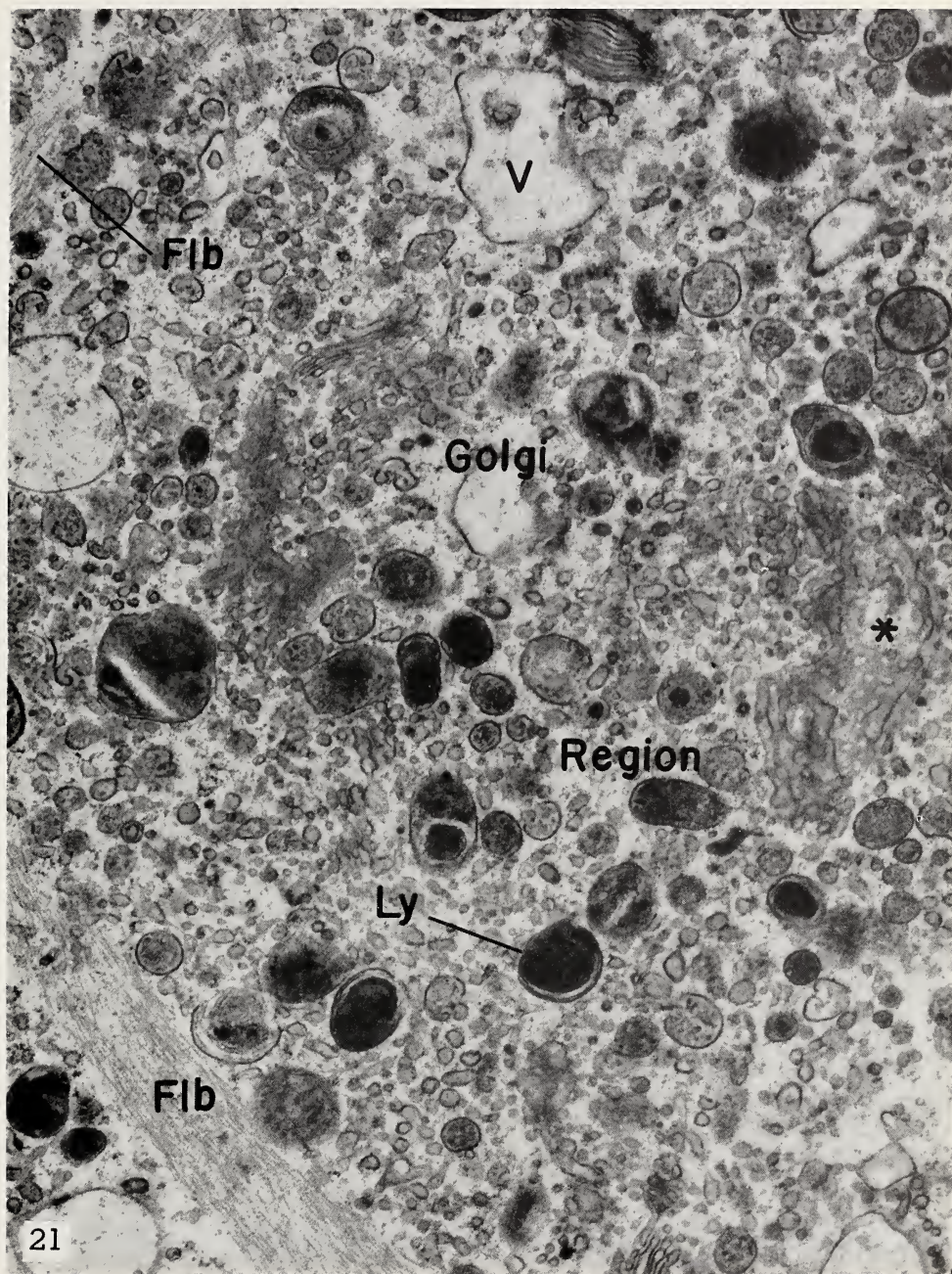


PLATE 23

FIGURE 22.—Epithelioid cell, 80-day culture. This micrograph demonstrates the boundary between the Golgi region (*bottom right*) and cytoplasm (*top left*). Several multivesicular bodies (MVB) are present within the Golgi region. Mitochondria (M) are closely associated (*arrow*) with cytoplasmic filaments (F1). Granular endoplasmic reticulum (Ger) is abundant. OsO_4 fixation; uranyl acetate and $\text{Pb}(\text{OH})_2$ stain. $\times 31,725$

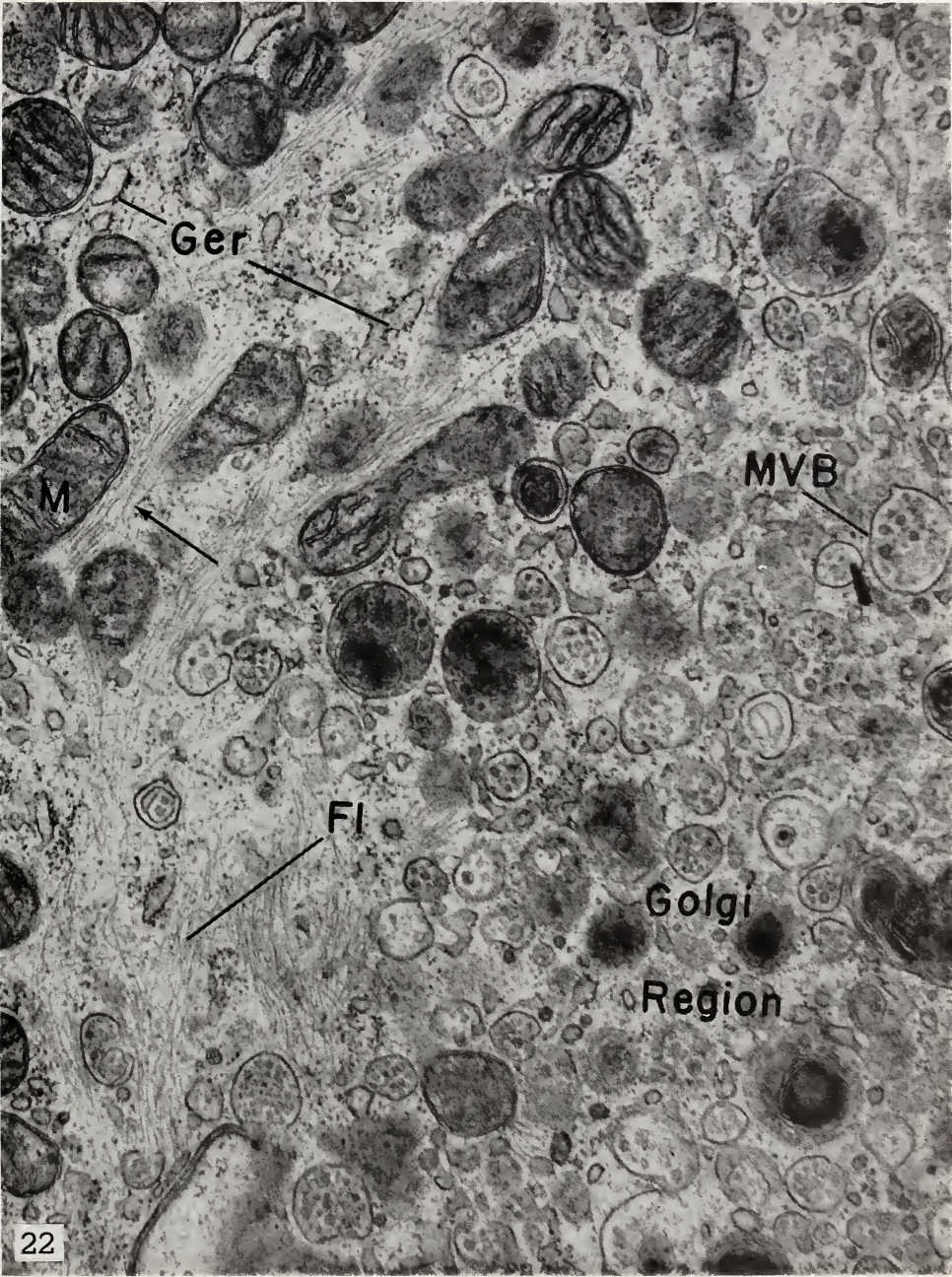
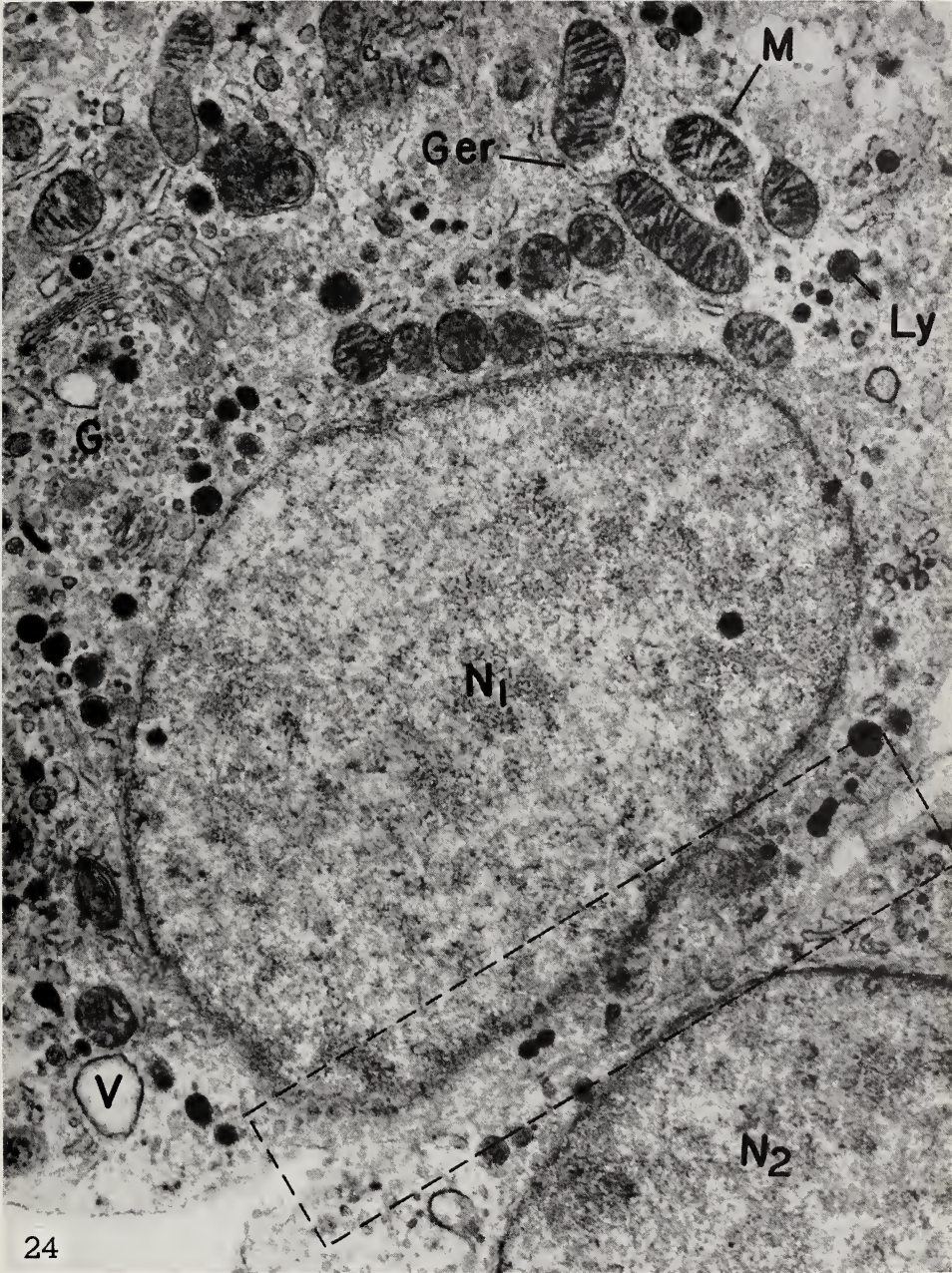




FIGURE 23.—Epithelioid cell, 80-day culture. Lysosomes (Ly) are especially prominent in the cytoplasm of this epithelioid cell. Smooth endoplasmic reticulum (Ser) and granular endoplasmic reticulum (Ger) are abundant. The large vesicular nucleus (N) shows a single nucleolus (Nu). OsO_4 fixation; uranyl acetate and $\text{Pb}(\text{OH})_2$ stain. $\times 23,200$



24

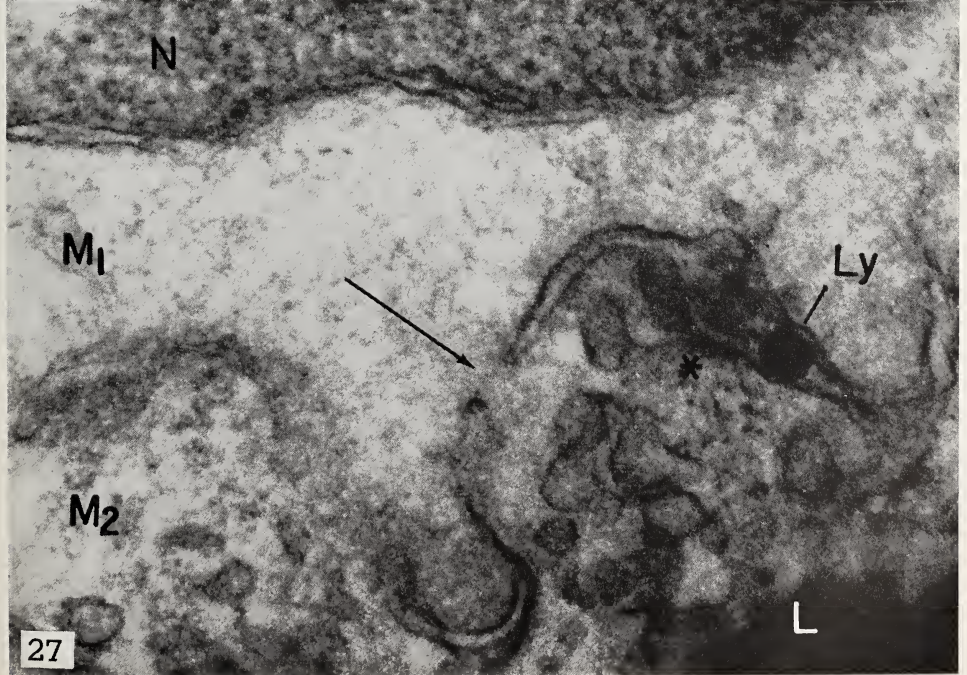
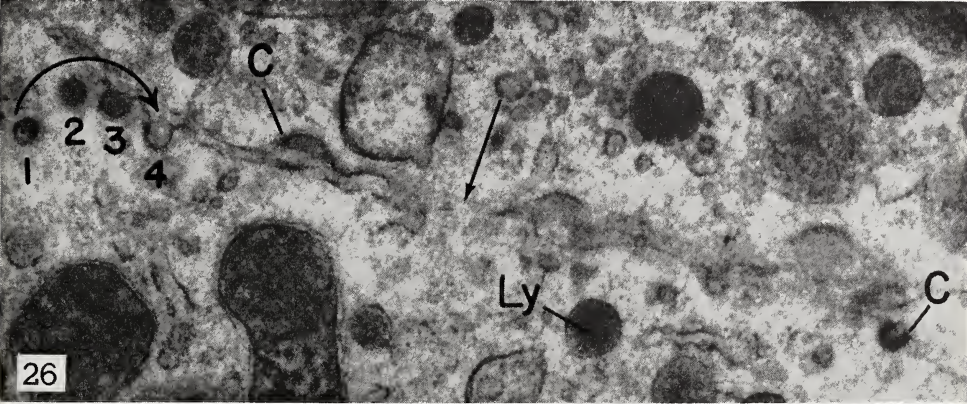
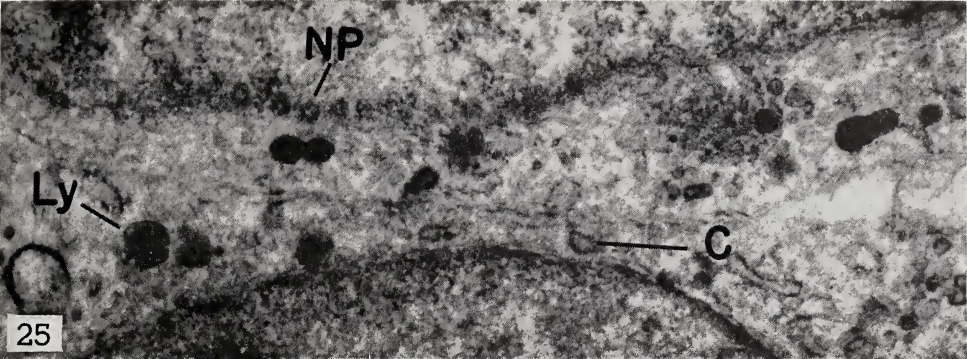
FIGURE 24.—Epithelioid cells, 7-day culture. These juxtaposed epithelioid cells (N₁ and N₂) are fusing their cell membranes (*region within dotted box*). OsO₄ fixation; KMnO₄ stain. $\times 20,175$

PLATE 26

FIGURE 25.—Epithelioid cell, 7-day culture. This micrograph shows the region of fusion in figure 24 at a higher magnification. "Fuzziness" of cell membranes in regions of fusion is often noted. Cup-shaped structures (C) and lysosomes (Ly) near region of fusion are a constant feature. OsO_4 fixation; KMnO_4 stain. $\times 31,400$

FIGURE 26.—Epithelioid cells, 7-day culture. This region of fusion at high magnification shows a bridge (*arrow*) of cytoplasmic continuity between two cells. *Note* the numerous cup-shaped structures (C) in both cell membranes and numerous lysosomes (Ly) nearby. *Semicircular arrow* and *numbers 1, 2, 3, and 4* demonstrate a plausible sequence for discharge of small granules at the cell membrane. Number 3 of this clockwise series appears to be halfway into the extracellular space between the cells. Number 4 appears empty. Each of these four structures measures 1100 Å in diameter. OsO_4 fixation; KMnO_4 stain. $\times 36,050$

FIGURE 27.—Monocytes, 24-hour culture. This micrograph demonstrates a region of cytoplasmic continuity (*arrow*) between two monocytes. A small granule (Ly), measuring approximately 950 Å in diameter, is in the intercellular space. The dense, cupped portion of membrane nearby (*) most likely represents its former bounding membrane fused with the cell membrane. OsO_4 fixation; KMnO_4 stain. $\times 83,600$



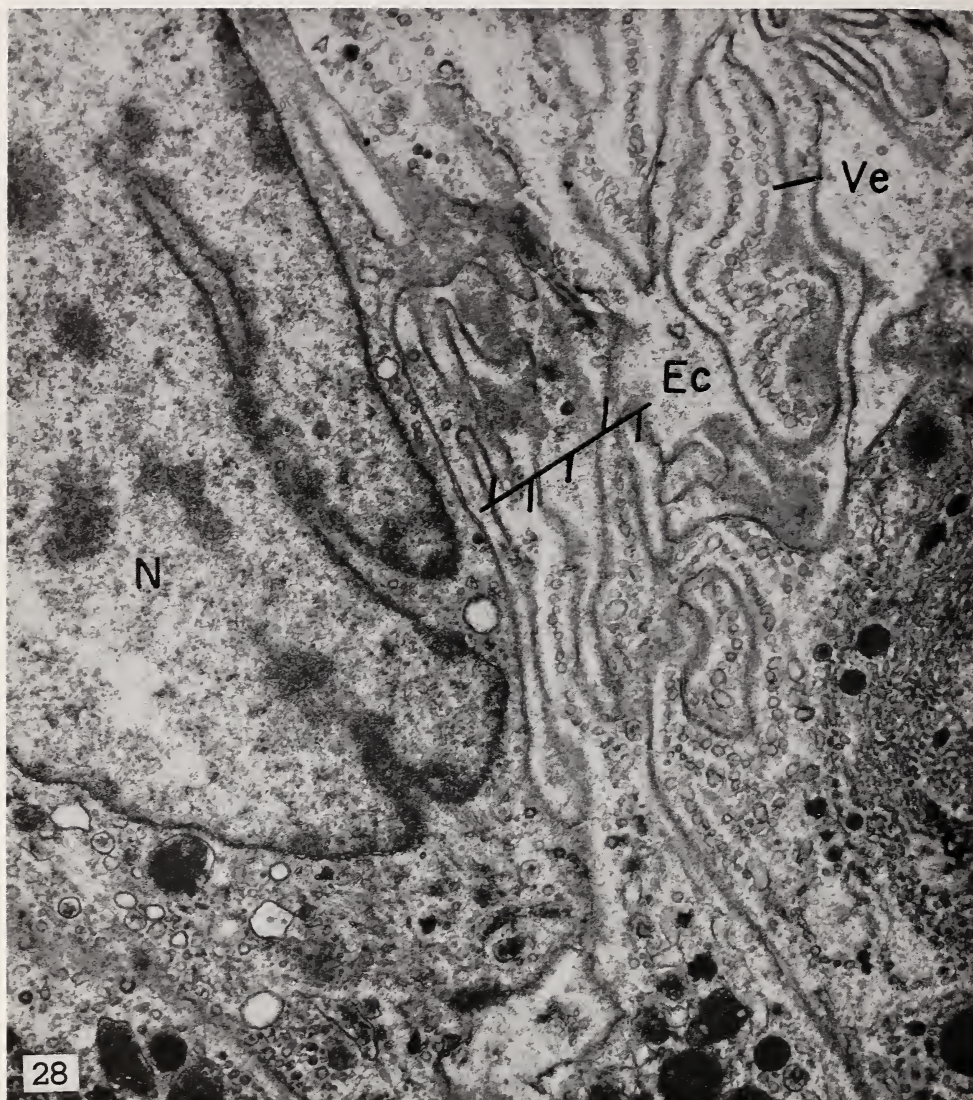


FIGURE 28.—Epithelioid cells, 15-day culture. These contiguous epithelioid cells display multiple ectoplasmic interdigitations (Ec). Rows of vesicles (Ve), delineating filiform areas of ectoplasm, suggest cytoplasmic continuity may be achieved by vesiculation of parallel apposed membranes, such as occur here in regions of reciprocal interdigitations. OsO_4 fixation; KMnO_4 stain. $\times 17,550$

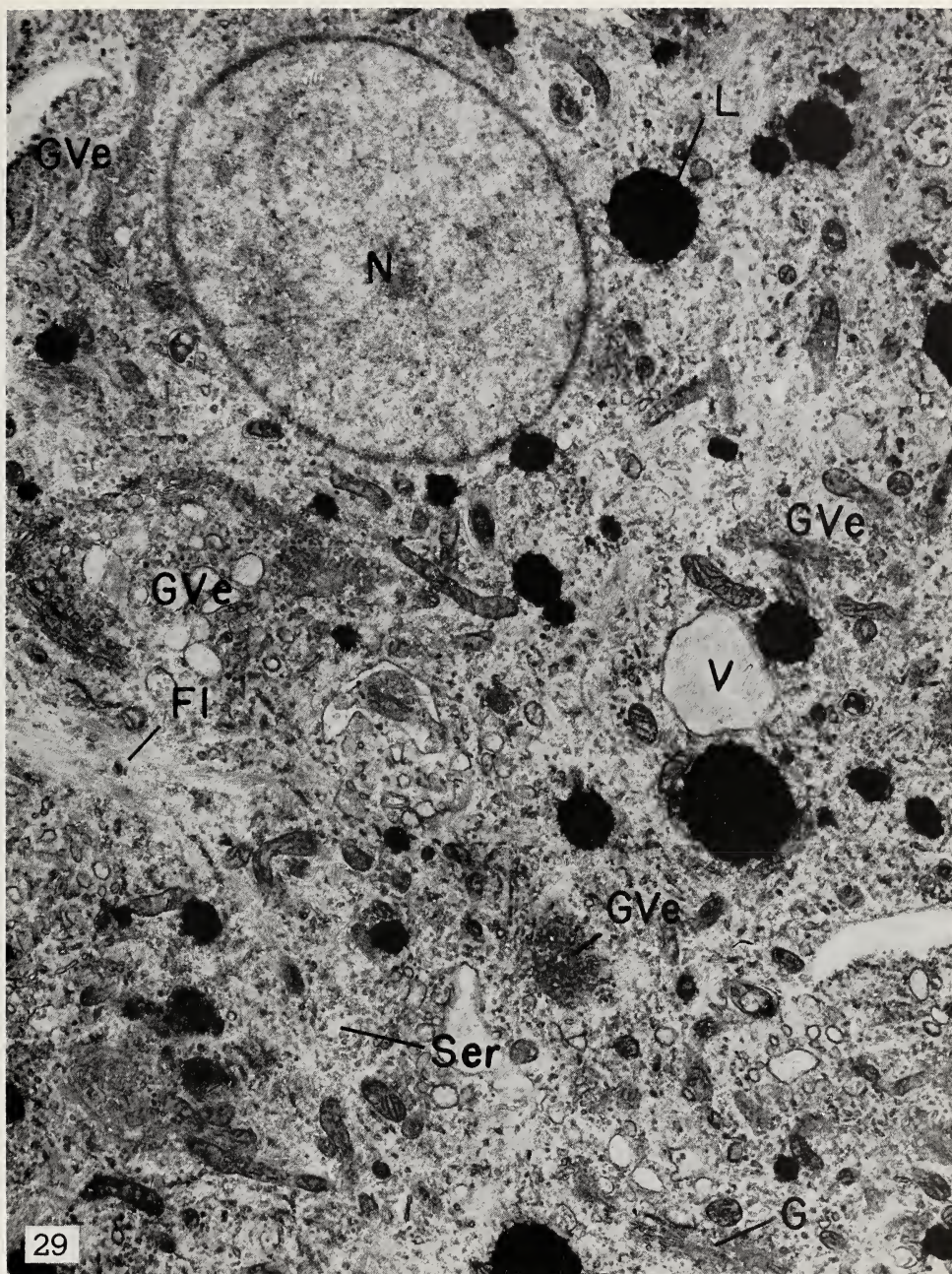


FIGURE 29.—Giant cell, 70-day culture. This small area demonstrates a single typical nucleus (N) ringed by several small collections of Golgi vesicles (GVe). Mitochondria and smooth endoplasmic reticulum (Ser) are abundant. *Black areas* represent lipid droplets (L). A small collection of filaments is present (Flb). OsO₄ fixation; KMnO₄ stain. $\times 13,200$

SUTTON

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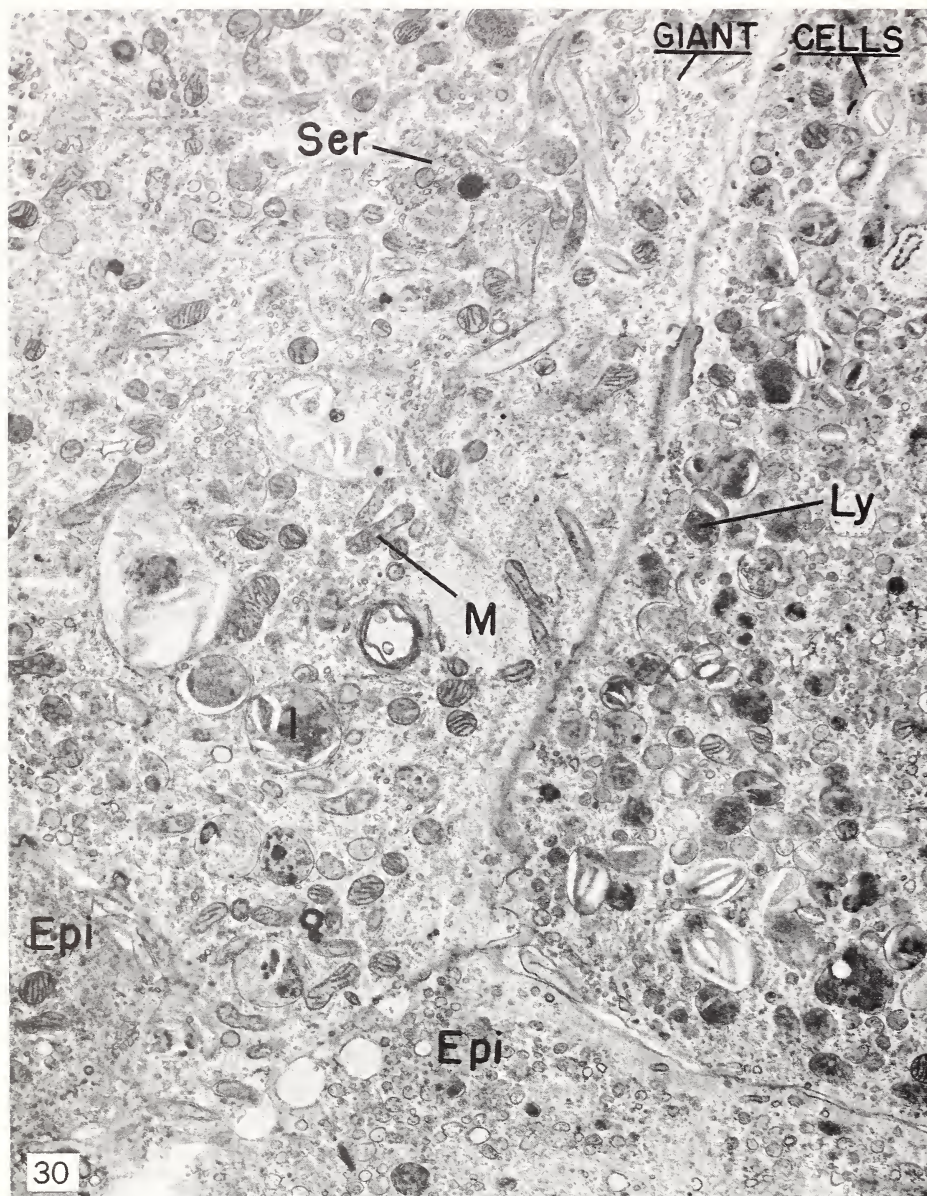


FIGURE 30.—Giant cell, 80-day culture. This field includes portions of contiguous giant cells and two epithelioid cells (Epi). Note predominance of smooth endoplasmic reticulum (Ser), mitochondria (M), and large cytoplasmic inclusions (I), in giant cell on left. Giant cell on right contains a large number of lysosomes (Ly). OsO_4 fixation; uranyl acetate and $\text{Pb}(\text{OH})_2$. $\times 6,300$

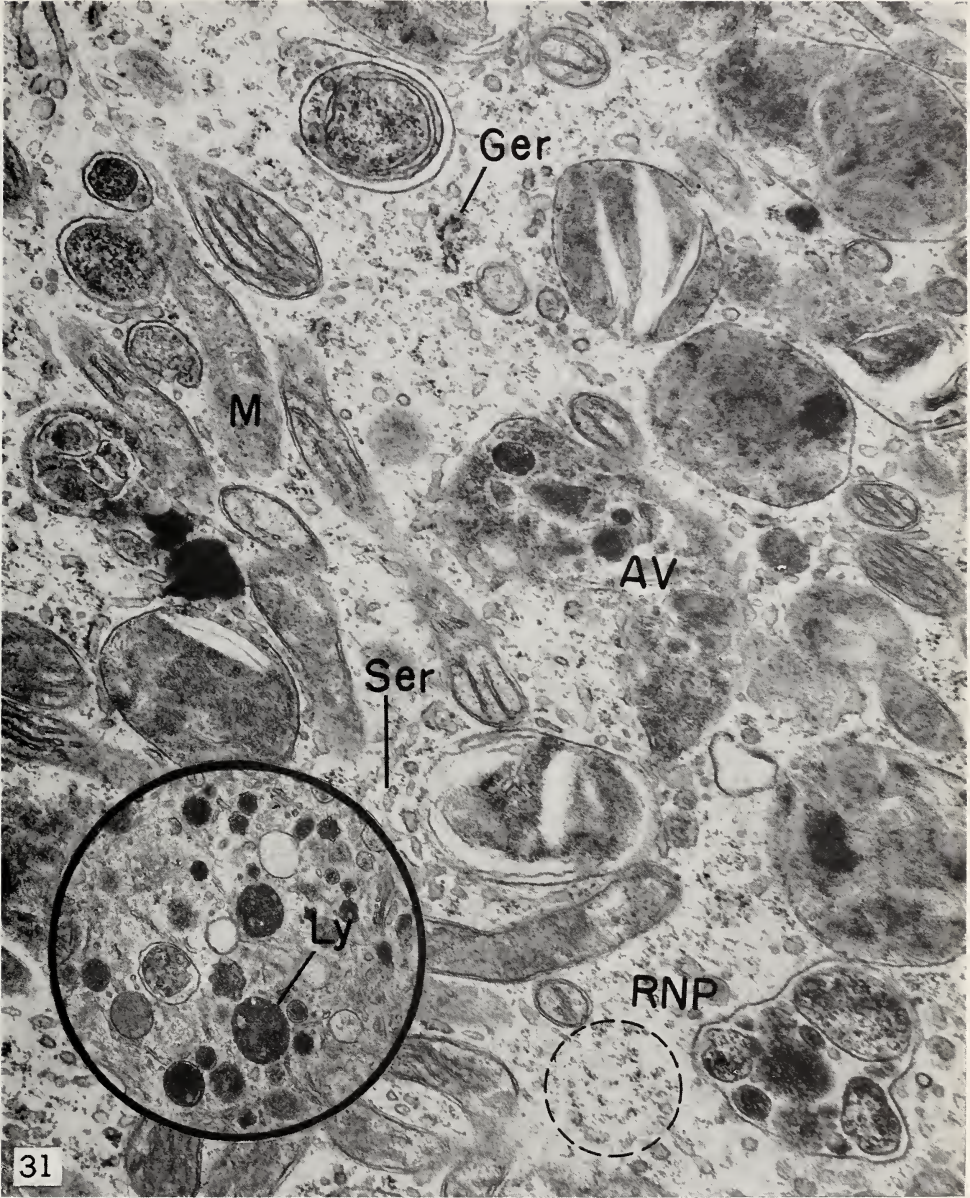


FIGURE 31.—Giant cell, 80-day culture. This area of cytoplasm contains several large cytoplasmic inclusions, many appearing to be autophagic vacuoles (AV). Granular endoplasmic reticulum (Ger) and RNP particles (RNP and *dotted circle*) are abundant. *Inset*: Lysosomes (Ly) from a 26-day-old giant cell. OsO_4 fixation; uranyl acetate and $\text{Pb}(\text{OH})_2$ stain. $\times 26,850$; inset $\times 13,325$

PLATE 31

FIGURE 32.—Giant cell, 11-day culture. These densely packed filamentous mitochondria from a small area of a multinucleated giant cell show multiple branching points (*arrows*). The majority are tangentially sectioned; the remainder show an orthodox internal structure. *Inset* demonstrates a large bundle of 100 Å filaments (Flb) beside a mitochondrion with an attenuated outer membrane (Om). Glutaraldehyde post OsO₄; lead citrate. $\times 44,650$; *inset* $\times 43,500$

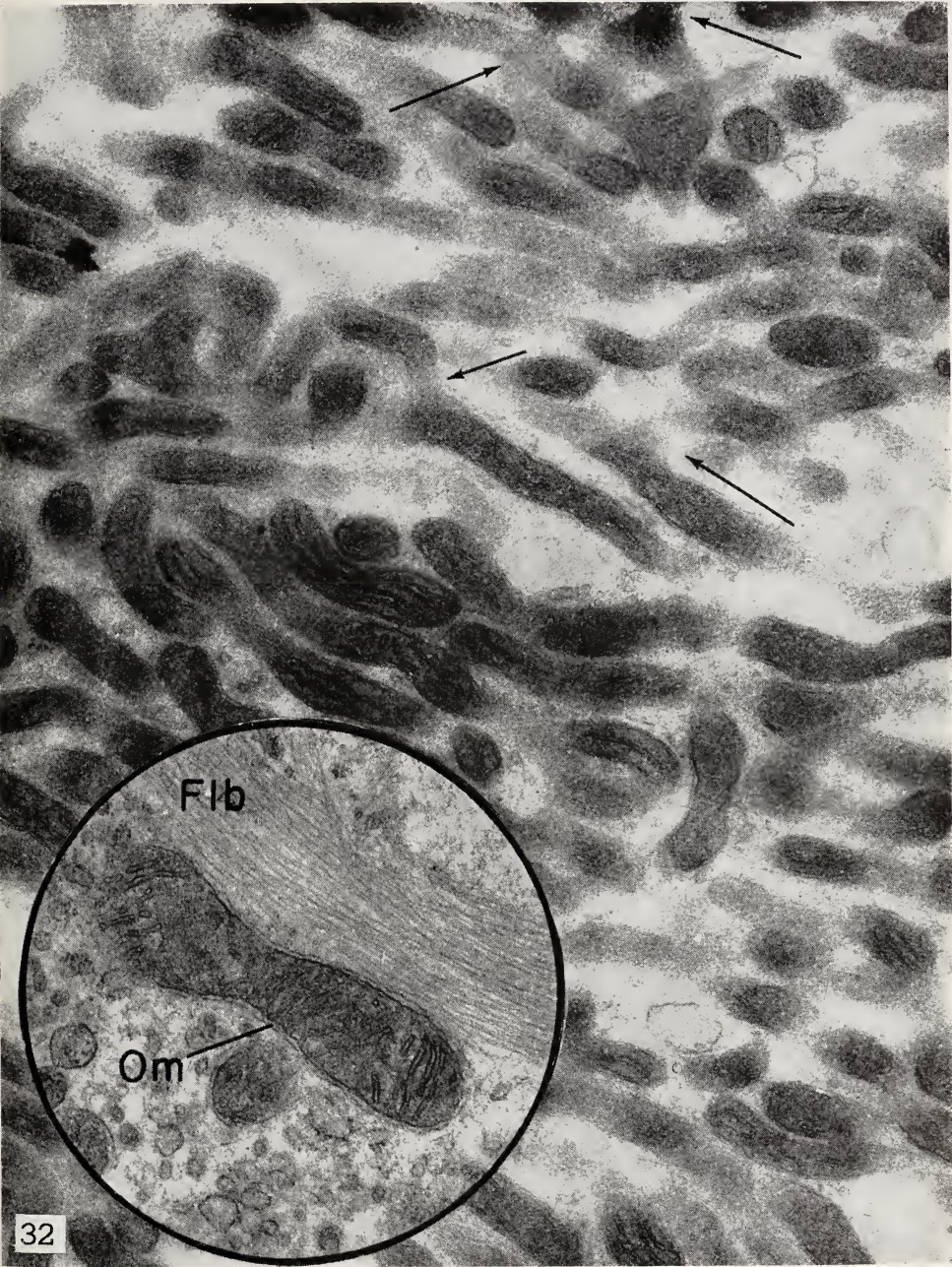


PLATE 32

FIGURE 33.—Giant cell, 161-day culture. Branching smooth endoplasmic reticulum (Ser) predominates in this portion of this large giant cell. The nucleus (N) has a single nucleolus (Nu) and a very narrow band of heterochromatin (Cr). Filaments (Fl and Flb) are numerous. OsO_4 fixation; KMnO_4 stain. $\times 25,300$

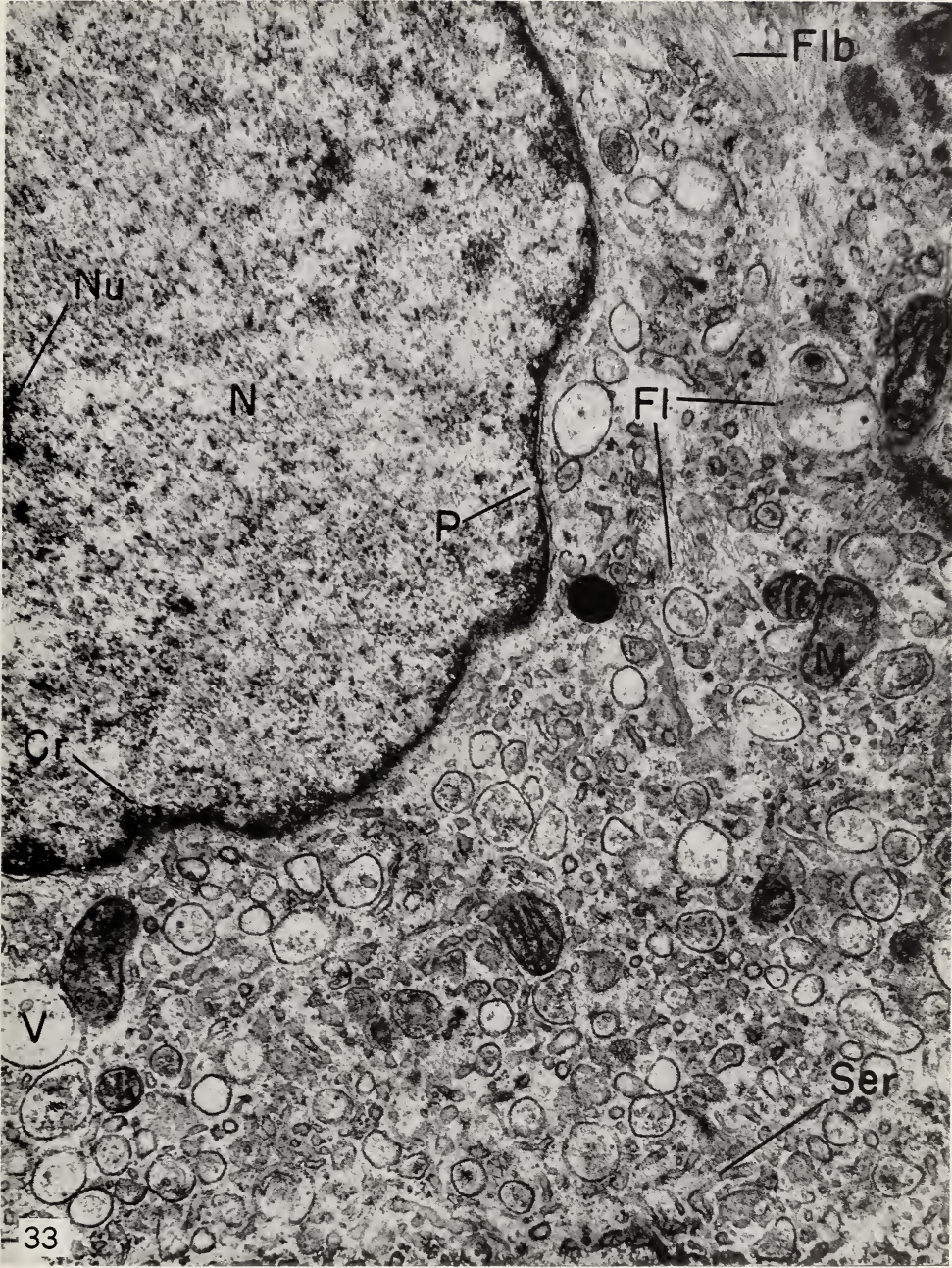


PLATE 33

FIGURE 34.—Summary of ultrastructural changes in tissue culture.

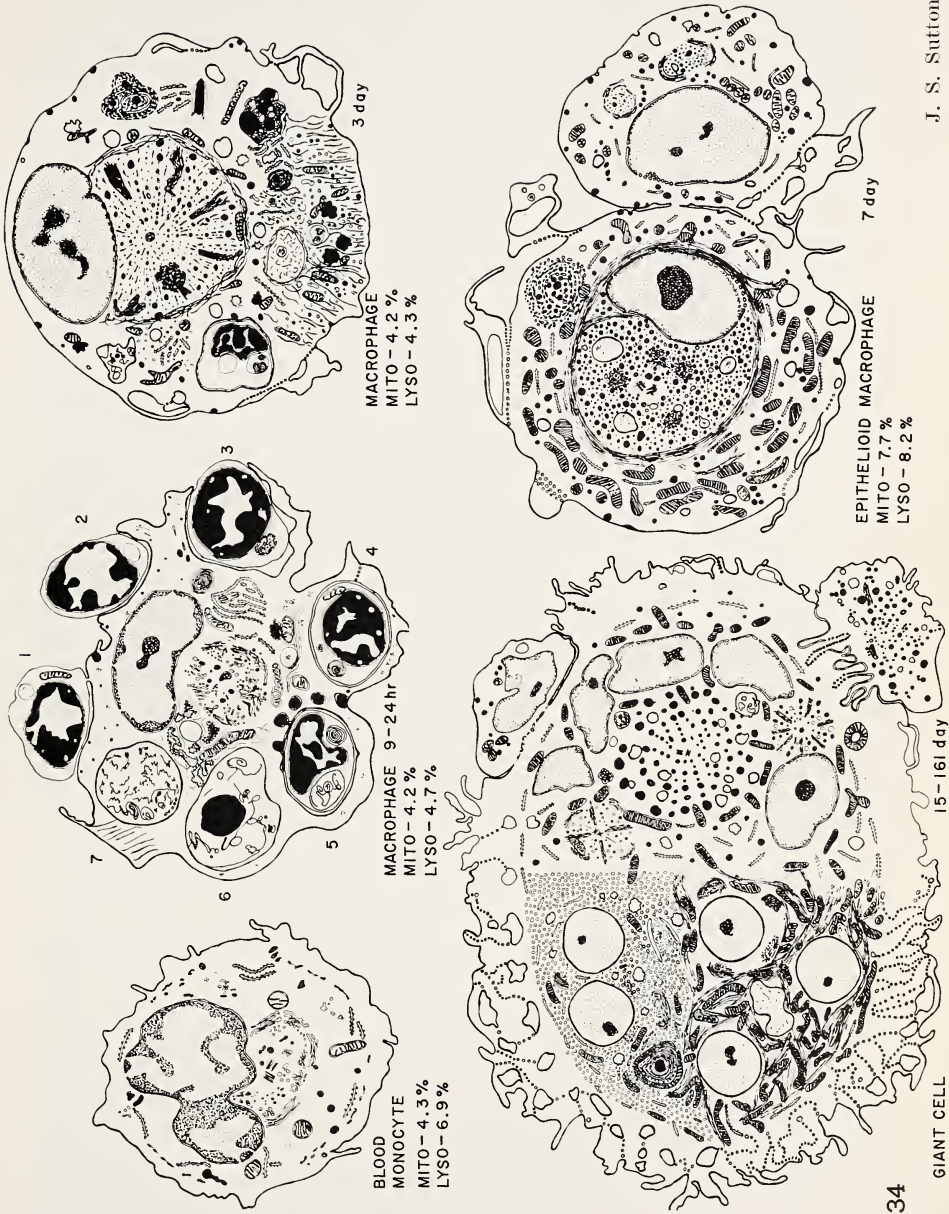
This diagram summarizes major ultrastructural changes and should be examined in conjunction with the summary. Cell stages summarized are in clockwise sequence: blood monocyte; macrophage, 9–24 hours and 3 days; epithelioid macrophage, 7 days; and giant cells, 15–161 days. No identifying labels are attached to the organelles since they are self-explanatory. The clockwise sequence around the 9–24-hour macrophage represents sequential stages observed during phagocytosis and digestion of a blood platelet, or thrombocyte. The epithelioid cells depicted at 7 days represent two typical cells undergoing fusion of their cytoplasmic membranes. The giant cell shows two attached epithelioid cells: The one at *upper right* sits in a depression on the cell surface, and the one at *lower right* is attached to the giant cell by cytoplasmic interdigitations.

Increase in cell size is noted. Blood monocytes average $6.0\ \mu$ in diameter; macrophages, $9\text{--}12\ \mu$; epithelioid cells, $30\text{--}40\ \mu$; and giant cells, $100\ \mu$ or more. The Golgi region increases in size and complexity and eventually contains massive accumulations of acid-phosphatase-positive granules.

In blood monocytes 4.3% of the cytoplasmic volume (S1) is occupied by mitochondria; 6.9% by lysosomes. In macrophages at 24 hours, the percentage of mitochondria remains the same, whereas lysosomes are reduced to 4.7%. It is interesting that the fraction of cytoplasmic volume occupied by these two organelles tends to be almost equal. In macrophages at 3 days, mitochondria account for 4.2% and lysosomes for 4.3% of the cytoplasmic volume. At 7 days mitochondria total 7.7%, lysosomes 8.2%. Also, quantitative measurements reveal approximately a threefold increase in the amount of endoplasmic reticulum between the blood monocyte and epithelioid cell at 7 days.

Cytoplasmic filaments are rare in monocytes. Macrophages have a loose network in their cytoplasm. They greatly increase in concentration in epithelioid cells, in which an annular bundle often encircles the cytocentrum.

J. S. Sutton



DISCUSSION

Dr. Porter: Thank you, Dr. Sutton, for this remarkable story. It is not easy to get this kind of information from cultured cells. These objects are difficult to fix and to handle technically. We should congratulate this man on his achievement.

Dr. Sutton: Thank you very much.

Dr. Fawcett: In one's scientific career, it is always particularly gratifying when some observations of earlier years, which seemed quite trivial at the time, later take on new significance. I derived this special kind of enjoyment from Dr. Sutton's paper.

About 15 years ago, inspired by the studies of Duncan Hetherington, Fritz Jacoby, and others, Dr. Leon Weiss and I undertook studies of monocyte-macrophage transformation, using methods very similar to those Dr. Sutton has employed, but using the light microscope. We reported that one of the most striking early changes was the development of a strong acid phosphatase reaction in the Golgi region. This seemed interesting, though we didn't quite know why, because at that time acid phosphatase was just one of several "enzymes in search of a function" for which histochemists were then enthusiastically providing new methods. In the years since then, the lysosome was invented by de Duve and his co-workers and shown to contain an assortment of hydrolytic enzymes, of which acid phosphatase is only one.

At a recent meeting in Japan, Dr. Eichi Yamada presented histochemical evidence, through the electron microscope, for the synthesis of peroxidase in the granular reticulum of neutrophilic and eosinophilic myelocytes, and for its concentration in the Golgi apparatus into the specific granules which are, of course, a form of lysosome.

Dr. Sutton has just presented evidence for the elaboration of lysosomes in the Golgi apparatus during the monocyte-macrophage transformation. So it is gratifying to me to hear about this work, which begins now to give some functional significance to our own observation of enhanced acid phosphatase in the Golgi of stimulated monocytes. It appears this is associated with the elaboration of lysosomes that the macrophage can use in its phagocytic business.

The evidence, I think, seems quite good, from what we have seen here, that the lysosomes develop in the Golgi complex like other secretion granules. Their function within the cell by coalescence and discharge of their enzymes into heterophagic vacuoles is fairly well documented in the work of Cohn and Hirsch and others. It is commonly assumed that lysosomal enzymes may leave the cell and act in the extracellular matrix.

An example familiar to this audience would be in the vitamin A experiments of Dame Honor Fell and Lewis Thomas, where proteases, presumably of lysosomal origin, were believed to be responsible for the dramatic resorption of the cartilage matrix. But in this and other examples of extracellular action of lysosomal enzymes, the conditions are usually such that the permeability of the lysosomal membrane and the cell membrane could be expected to be altered by autolysis, ultraviolet radiation, or an exogenous surface-active agent such as vitamin A.

One of the novel features of Dr. Sutton's paper is his suggestion that the lysosomes may secrete their enzymes at an undamaged cell surface in the same way a glandular cell releases its products. This is interesting and provocative, but the morphological evidence is, I believe, not entirely convincing. And I gather that Dr. Sutton himself puts it forward as a tentative interpretation.

He referred to the small, cuplike structures on the surface of his cells, and suggested that these might be stages in the release of lysosomal contents at the cell surface. These appear to me to be very much more like the so-called coated vesicles that have been described in a great variety of cell types. Porter and Rolf described them in the *uptake* of proteins in relation to the development of the mosquito

ovocyte. Bessis has described them in cells of the erythrocyte series, and we have described them in a number of places.

I would suggest that the smaller structures Dr. Sutton showed at the surface of his macrophages and epithelioid cells were of this nature rather than a stage in the release of lysosomes at the cell surface.

Dr. Sutton: I am not going to offer much rebuttal about these cups in the cell membrane. Granules and vesicles of similar dimensions are always seen in and near the cell membrane at this stage of culture. There is evidence that acid phosphatase is present in culture fluid. It is definitely a possibility that lysosomal granules do empty their contents at the cell surface.

Dr. Fell: I want to take up the point Dr. Fawcett raised about the release of lysosomal enzymes. My biochemical colleague John Dingle and I have found that, where organ cultures are concerned, you always get a certain release of acid phosphatase and acid protease without the addition of any agent. This release can be considerably reduced by a very small dose of hydrocortisone and much increased by a very small dose of vitamin A.

Just recently we found that the biggest release associated with a greatly increased synthesis is produced by sucrose, which stimulates endocytosis, fills the cells with vacuoles, and stimulates them to produce an extraordinary amount of lysosomal enzyme that does not affect in any way lactic acid dehydrogenase, which remains the same.

Miss Audrey Glauert has been studying the electron microscopy of this sucrose effect and finds that the cells look extraordinarily active and healthy. They also undergo mitosis at quite a high rate. We suspect that the release may not have exactly a damaging effect on the membrane of the lysosomes in these particular circumstances.

Dr. Fawcett: Thank you, Dr. Fell, for these very interesting comments. It is certainly true that the hydrocortisone and vitamin A effects are effects on the membrane. I do not resist the notion that the cells may actively release these enzymes, but I do think the morphological evidence has not been very convincing.

Dr. Fell: No, we don't really know how they get out. We can only guess that there is a fusion of vesicles between the outer membrane, that they don't just seep through the cytoplasm.

Dr. Jacoby: I have a few questions for Dr. Sutton. The first concerns the formation of binucleated cells. There is considerable proliferation in chick macrophages, and I wonder whether you have any observations on this process, since mitosis does occur outside the plasmic divisions. We think that all these binucleated cells occur by fusion. It is very likely that the giant cells do. But whether the first binucleated cells do so is, in my opinion, rather doubtful.

The other point in which I am interested, although I know how difficult it is to interpret, concerns the mitochondria. I wonder whether you have seen any evidence of fission of mitochondria, either in dividing cells or in those cells which aggregate. Of course, in these multinucleated giant cells, the great aggregation is due to the many cells that come together, so you get an enormous population of mitochondria. It would be interesting to find out whether you have any evidence of the way in which mitochondria multiply.

Finally, there is a point on the fusion of cells. Are there any desmosome-like structures or do they never occur? When cells are spread out in this absolute fashion, they still seem to retain their individuality and sometimes even show they are not really fused. These are epithelial membranes, but surely they have the individuality under which the membrane can actually be seen to float away.

In spite of the wonderful preparations Dr. Sutton showed us, I think we probably get only a fraction in a cut of these very ramifying types of cells and probably a great number of these complicated processes are actually lost in the process.

Dr. Sutton: I will take those questions in reverse. Desmosomal attachments have not been seen. When the membranes are closely apposed, there may be some thickening in the cytoplasm subjacent to the membrane, but there is never any desmosome formation.

Regarding your question about mitochondria, it is my impression that mitochondria form by division of previously existing mitochondria. But this is simply speculation. I see no forms of mitochondria within these cells that would appear to be constricting in a manner suggesting division. They may originate from cytoplasmic granules or possibly within the Golgi zone, but this problem awaits further study.

As far as the binucleated cells are concerned, I can't offer any answer.

Dr. Claude: I want to comment on Dr. Fawcett's presentation. I won't say anything more about the excellence of his preparations. We are quite used to that. I think it is, however, the best evidence thus far that the so-called nuclear pores have a physiological function in the activity of the cell. You have probably noticed, as I have, that there is a relation between the arrangement of the chromatin inside the nucleus and the pores. It seems that the chromosomes, or at least the amnion chromosomes, attach themselves around the nuclear pore. Maybe the pores are the reversal of that attachment or somehow in connection with it in channeling and conveying some of the chromosome activity toward the nuclear envelope.

As to the thickening from the innerface of the nuclear envelope, I have observed that this area only was reacting with adenosinetriphosphatase, which might indicate that the nuclear pores at least contain proteins. I think this is quite significant.

Dr. Gall: I would like to make one brief comment on Dr. Fawcett's presentation. Regarding the extrusion of material through the pores, his material is extremely similar to what is seen during oogenesis as first shown by Hollister and Ward and later by Miller. The only comment one could make is that this would indicate this is some kind of stable RNA which is being set out and probably is not a relationship to the shutting off of the nucleus, which doesn't occur in the ovocyte.

Dr. Porter: The stars are getting out of the constellations. I would like to ask Dr. Dalton to discuss whichever paper he prefers.

Dr. Dalton: I was stimulated by Dr. Fawcett's remarks to attempt to bring viruses into discussion, but I won't.

Over the past 10 or more years of looking at tissue culture material turned over to me by Virginia Evans and Katherine Sanford, I have been impressed by the fact that the material grown on chemically defined medium always looked esthetically nicer; the cells just had the things in them they were supposed to have, and did not contain many foreign bodies or phagosomes. However, in those containing horse serum or fetal calf serum things were a little different.

Strain L cells on chemically defined medium contain relatively few evidences of phagocytosis. The majority of formed bodies in the cytoplasm are mitochondria, whereas strain L cells in a medium containing horse serum contain a larger number of bodies, many of which are the result of phagocytosis. Lipid droplets are also more numerous in these cells.

Even in epithelial cell types like rabbit kidney, when grown in a medium containing calf serum, cytoplasmic bodies are present that represent phagocytosed material. These bodies are reduced when the cells are grown in media with reduced serum content.

My comments are primarily concerned with both Dr. Amos' and Dr. Sutton's presentations. My evidence suggests that the presence of serum alone or possibly stimuli from its presence may have something to do with the degree of phagocytosis that occurs.

I would appreciate Dr. Sutton's comments in regard to whether a comparison has been made by growing cells in the presence and absence of serum. There are two other minor points, Dr. Sutton. You mentioned that myelin figures were almost

characteristic of thrombocytes. Is this directly from peripheral blood or after they have been in tissue culture for a while? One other point: I may be wrong, but I got the impression that you used the term "multivesicular body" and "lysosome" as synonymous terms. Am I correct in that?

Dr. Sutton: It has been demonstrated that acid phosphatase is present within some of these small granules in multivesicular bodies. Therefore, that classifies it as a type of lysosome.

In regard to myelin figures: If these cells are taken directly from the bloodstream of the animal and fixed in OsO_4 this characteristic myelin figure is present in the cytoplasm.

In answer to your first question, I don't think there is any such thing as a "clean" macrophage. I have not tried to grow these cells in chemically defined media, but if it is true that serum stimulates phagocytosis, I wonder why these cells in the bloodstream, bathed in 100% serum, do not phagocytize more than they do! I have grown all of these cells in autologous or homologous sera to simulate as nearly as possible an *in vivo* environment. I have not used heterologous sera. I tried it when I first started and the cells didn't grow.

CHROMOSOMES AND GENETIC EXPRESSION OF CELLS IN CULTURE

Chairman: Dr. T. C. Hsu, M. D. Anderson Hospital, Houston, Texas

Speakers: Dr. Henry C. Pitot, McArdle Memorial Laboratory, Madison, Wisconsin
Dr. Stanley Gartler, University of Washington School of Medicine, Seattle, Washington

Panelists: Dr. Margery Shaw, University of Michigan, Ann Arbor, Michigan
Dr. Robert Schimke, Stanford University, Palo Alto, California
Dr. Hans Laufer, University of Connecticut, Storrs, Connecticut

Dr. Hsu: I think the studies of genetics follow a rather logical sequence. During the last century since Mendel, the studies have been primarily on the mode of inheritance from parent generations to filial generations. When this was understood the studies went on to the nature of the genetic material.

This is the present boom in molecular biology. How is the gene made up chemically? How does it express? And in so doing, naturally, the simpler the material, the simpler is the task. People went from *Neurospora*—that is too complicated—to *Escherichia coli*—still a little too complicated—to MS-2.

One thing, MS-2 cannot answer some of the questions we have. How is the genetic material controlled in the higher organisms? How, in the cell that has all the genetics material, do the messages for the brain, liver, kidney, or skin develop into an organism containing all these, yet not expressed? The next step, therefore, is studies on developmental biology. The important portion of this developing biology is to study the somatic cell genetics. Tissue culture certainly made a great contribution to this area in the last decade.

A number of developments and improvements made this contribution possible, including the cell culture technique, the simplification of it, the cell nutrition from which we can grow cells, the simplification of the cloning technique in which we can obtain single cell colonies, the techniques of the study of chromosomes, the genetic apparatus, and, finally, the techniques of cell hybridization.

All these are just the beginning. We are witnessing here some of the fruits of this type of study, which we hope eventually will naturally flourish into real understanding of how differentiation comes about.

Control of Biochemical Expression in Morphologically Related Cells *In Vivo* and *In Vitro*^{1, 2}

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SUMMARY

The control of genetic expression in differentiated mammalian tissues is rapidly becoming one of the major areas of biochemical investigation. Patterned after the microbial system, enzyme induction and, most recently, enzyme repression have been found to occur in mammalian tissues both *in vivo* and *in vitro*. In rat liver, tryptophan pyrrolase, tyrosine transaminase, and glutamic-pyruvic transaminase have been induced by glucocorticoid administration *in vivo* whereas serine dehydrase has been induced by a complex mixture of amino acids. The latter enzyme may also be repressed by glucose or induced by the hormone, glucagon. Multiple controls of a single enzyme have also been shown for tryptophan pyrrolase. The major differences seen in the control of genetic expression in multicellular as opposed to unicellular organisms appear to reside in the disposition of messenger RNA templates and the control of enzyme synthesis at the translational level. When cells are cultured *in vitro*, major environmental changes occur which are

in part followed by major changes in enzymology. A number of examples of metabolic controls in cultured mammalian cells have been described but the relationship of control mechanisms seen *in vitro* to those operative *in vivo* has been investigated in only a relatively few cases. Such a system is that of the highly differentiated hepatocellular carcinoma, the Reuber H-35 which still exhibits several mechanisms controlling enzyme levels *in vivo* but only a relatively few *in vitro*. The karyotype of this cell line has changed from an original mode of 43 *in vivo* to 46 *in vitro* and most recently to 102. The hormonal control of tyrosine transaminase is operative *in vivo* and *in vitro*, but the multiple substrate control of serine dehydrase is apparently lost *in vitro*. The marker enzyme, ornithine transaminase, is present at relatively high levels both *in vivo* and *in vitro*. The biochemistry of this tumor may also be compared with that of the Novikoff and Dunning hepatomas which are relatively poorly differentiated but still retain certain marker enzymes

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both *in vivo* and *in vitro*. It thus appears that in selected neoplasms certain biochemical expressions may be retained intact both *in vivo* and when the cells are cultured *in vitro*.—Nat Cancer Inst Monog 26: 145–166, 1967.

THE ENVIRONMENTAL control of cellular metabolism is a topic that has fascinated biochemists throughout this century. However, only recently has our knowledge of the chemistry of living matter grown to sufficient sophistication to allow us to study the mechanisms of cellular regulatory function. Perhaps the highest expression of the phenomena of cellular regulatory mechanisms is seen in the mammalian organism wherein a multicellular multiclonal organism must not only cope with the environment in which it lives but also must have delicately balanced mechanisms regulating an internal intercellular environment in which the cell colonies survive for their mutual benefit. Furthermore, unlike the unicellular organism whose only battle for survival is with the external elements, the mammalian organism consists predominantly of tissues and cells in the resting phase or interphase of their growth.

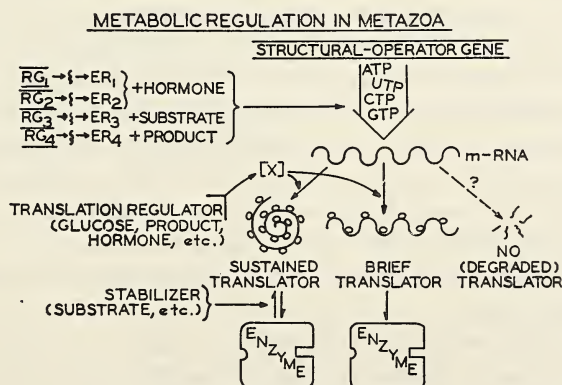
The importance of the maintenance of this complicated internal environment has been forcefully emphasized by the complexity of the semisynthetic environments used in the cultivation of cells outside the mammalian organism (1). The radical morphologic and biochemical changes accompanying the transition from *in vivo* to *in vitro* environments have necessitated consideration of the similarities and differences between the natural intercellular environment and that of the tissue culture media. Although those difficulties are of major importance, it will not be the purpose of this paper to consider them directly but rather only as they affect the control of specific biochemical processes *in vivo* and *in vitro*. Herein we will be concerned with mechanisms controlling biochemical expression of tissues *in vivo* as compared with the same or related tissues cultured *in vitro*.

CONTROL OF GENETIC EXPRESSION IN MAMMALIAN TISSUES *IN VIVO*

In text-figure 1 is depicted a theoretical scheme for the regulation of genetic expression in tissues of multicellular organisms. The basis of this format is the operon concept of Jacob and Monod (2) which is based primarily on studies in microorganisms. The RG_1 through RG_4 are regulator genes according to the operon concept. These may interact with hormones or substrate or product to affect the rate of synthesis of messenger RNA (mRNA). The primary alterations between this scheme and that of the microbial systems may be seen at the translational level in

which the messenger RNA has three distinct possible fates. It may be rapidly degraded and not translated at all or it may be briefly translated as is seen in microbial systems (2). The most important difference between unicellular and multicellular organisms appears to be the presence of what may be termed sustained translators or stable messenger RNA templates which can exist and maintain translational processes even throughout the lifetime of the organism. Such a condition implies that control mechanisms exist at the translational level. Furthermore, as Schimke and his co-workers have shown (3), an enzyme synthesized from a stable template such as tryptophan pyrrolase may itself be stabilized and its endogenous breakdown prevented by the substrate of the enzyme. Not depicted on this scheme are the many examples of regulation at the enzyme level itself by positive and negative feedback (4).

The first demonstration of net enzyme induction in mammalian organisms was described by Kenney in the liver enzyme, tyrosine- α -ketoglutarate transaminase (5). By utilizing immunochemical techniques to study the incorporation of labeled amino acids into the enzyme molecule *in vivo*, Kenney was able to show that the administration of glucocorticoids to an animal resulted in a rapid increase in the rate of synthesis of the enzyme. Later studies by Greengard *et al.* (6) showed that the antibiotic, actinomycin D, an inhibitor of DNA-directed RNA synthesis, when administered just prior to the glucocorticoid, completely suppressed enzyme induction. These data indicated that the increased rate of enzyme synthesis depended on the synthesis of new messenger RNA molecules. In related studies these authors were able to demonstrate that with the liver enzyme, tryptophan pyrrolase, only the hormone-stimulated increase was inhibited by actinomycin D as opposed to the substrate-stimulated increase which appeared to be completely resistant to this antibiotic.



TEXT-FIGURE 1.—Regulation of genetic expression in tissues of multicellular organisms.
See text for explanation.

More recent work by Schimke and co-workers (3) showed that not only were the hormonal and substrate inductions of tryptophan pyrrolase in the adrenalectomized animal different in their sensitivity to this antibiotic, but also the kinetics of induction by the two methods was quite different. The hormone stimulated a complex third- or fourth-degree type of curve whereas tryptophan given to the adrenalectomized animal resulted in a virtual straight line increase in the level of the enzyme, the slope of the line being equal to the half-life of the enzyme. On this basis these workers suggested that the hormonal stimulation of enzyme activity reflected a true enzyme induction resulting from an increased rate of synthesis of the enzyme, whereas the tryptophan-stimulated increase was due to the stabilization and prevention of breakdown of the enzyme by the usual process of intracellular protein turnover. Immunochemical studies (3) confirmed these suggestions and thus elegantly demonstrated that the effect seen by Greengard with actinomycin D was a result of the effects on enzyme synthesis. These data also demonstrated that in the adrenalectomized animal the mRNA template for this enzyme must be extremely stable since it apparently was able to function without any continued renewal.

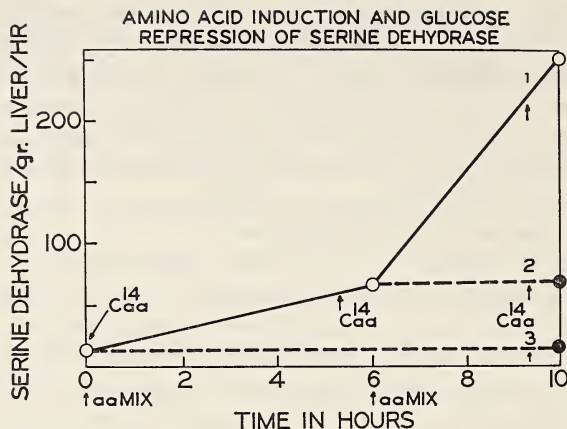
Studies in this laboratory in support of those of Wagner (7) have demonstrated that other controls for the enzyme tryptophan pyrrolase exist. Distal products of the pathways of the conversion of tryptophan to nicotinamide and its derivatives, *e.g.*, DPN, TPN, and related compounds, are capable of inhibiting the enzyme, tryptophan pyrrolase, in a manner compatible with an allosteric type of inhibition (8). Furthermore, in adrenalectomized animals the substrate induction of tryptophan pyrrolase is inhibited by the administration of nicotinamide or nicotinic acid but not by related compounds such as benzamide (8). The hormonal induction of the enzyme is only slightly affected by these compounds. This study performed by Dr. Yoon Sang Cho-Chung in our laboratory has also been done by using the immunochemical techniques described by Schimke and his co-workers, these data supporting those found by the measurement of enzyme activities. Thus, in the case of tryptophan pyrrolase in the intact rat, the control of the synthesis of the enzyme appears to be quite complex, involving hormonal induction, substrate stabilization, and distal product repression, the last two controls acting at the translating level. The phenomenon of multiple controls on a single enzyme is somewhat different from that seen in microorganisms where apparently multiple controls usually involve more than a single enzyme, *e.g.*, two distinct threonine deaminases and their role in threonine degradation and isoleucine biosynthesis (4).

Another enzyme, whose control has been rather extensively studied in our laboratory, is rat liver serine dehydrase. This enzyme is a relatively small molecule having a molecular weight of approximately 19,000–20,000 (9) and reportedly having several different catalytic functions. In earlier

studies (10, 11) we demonstrated that this enzyme could be induced to quite high levels by the forced feeding of casein hydrolysate to protein-depleted animals. Furthermore, this induction could be completely shut off by the simultaneous administration of glucose. The induction of the enzyme was at all times inhibited by puromycin but only inhibited early within the first hour or so after casein administration by actinomycin D. Actinomycin administered later than this time gave no effect or a slight stimulation to the induction of the enzyme.

On the basis of these studies it has been possible to find a period of time during which the induction of the enzyme is completely resistant to actinomycin and therefore its synthesis apparently proceeds in a manner very similar to that of the substrate stabilization of tryptophan pyrrolase. The period of time during which the enzyme is resistant to the antibiotic is approximately 6-8 hours and has been termed the template lifetime (12). Glucose repression occurs both during the periods of actinomycin D sensitivity and resistance. Thus the substrate stabilization of tryptophan pyrrolase as well as the nicotinamide repression of the enzyme and the glucose repression of serine dehydrase appear to be acting at the level of translation rather than transcription since the systems under study at that time are resistant to actinomycin D. In our laboratory Cho-Chung has already shown by immunochemical techniques that the nicotinamide repression does occur in the presence of this antibiotic.

It has also been possible to induce serine dehydrase by a mixture of synthetic amino acids (13). The data of text-figure 2 demonstrate the effect of the administration of a mixture of 5 amino acids at 0 and 6 hours on the level of the enzyme when given alone or in the presence of glucose from 0 time or of delayed glucose from the 6-hour point. It can be noticed that glucose very effectively inhibits any further increase in the level of the enzyme beginning from the point of initial glucose administration. Serine dehydrase has been purified in our laboratory by Dr. Jost, and antibodies were prepared which have been shown to be of a single species by immunodiffusion and immunoelectrophoresis. When pulse-labeled ^{14}C -valine is administered at the times shown in text-figure 2, the data of table 1 are obtained. During the 10-hour period of the experiment, there is a 25-fold increase in the level of the enzyme and at the same time there is a 25-fold increase in the incorporation of ^{14}C -valine into the enzyme protein. Glucose given from 0 time completely inhibits any incorporation of the amino acid into the enzyme. Furthermore, glucose given at 6 hours results in almost a complete suppression of enzyme synthesis as judged by the incorporation. Although there appears to be a doubling of amino acid incorporation, if one considers the total incorporation of amino acid into liver protein, 50% of the increased incorporation may be accounted for by the administration of the glucose. The percentage of total incorporation of amino acid attributable to serine dehydrase is about



TEXT-FIGURE 2.—The effect of injection of an amino acid mixture (phenylalanine, threonine, histidine, isoleucine, and tryptophan) on the activity of rat liver serine dehydrase. A dose of 0.2 g of an equal molar mixture of the 5 amino acids in 4 ml of isotonic saline at a pH of 7.1 were intubated at 0.0 time and again at 6 hours. In those animals receiving glucose, 1.0 g of glucose was also intubated with the amino acids at 0 and 6 hours, or only at 6 hours in the case of the delayed glucose administration. For the data of table 1, 12 μ c of ^{14} C-L-valine was injected intraperitoneally at the times indicated on the graph and the animal was killed 30 minutes later. (Data of Jost and Pitot, *Fed Proc* 25: 341, 1966.)

1/10 of 1%. In contrast, when one performs the same experiments on the Morris 5123 hepatoma, the incorporation into the very high level of serine dehydrase seen in the tumor amounts to almost 4–5% of the total incorporation of amino acid into protein.

In addition to the induction of serine dehydrase by amino acids, this enzyme may also be induced by the gluconeogenic hormone, glucagon (11).

TABLE 1.—Induction of serine dehydrase by amino acid mixture (phenylalanine, threonine, histidine, isoleucine, tryptophan) and its repression by glucose*

	Enzyme activity (units/g liver)	^{14} C-valine incorporation (dpm/g liver)	
		Serine dehydrase	Soluble protein
0 time control (5)†.....	4.5	75.5	320,588
6 hours+AA only (6).....	60.0	836.0	
10 hours+AA only (7).....	125.6	1,850.0	504,957
10 hours AA+glucose (5).....	5.4	50.5	323,927
10 hours AA+delayed glucose at 6 hours (4).....	45.0	150.0	489,417

*See legend of text-figure 2 for format of the experiment. Data taken from those of Jost and Pitot, *Fed Proc* 25:341, 1966.

†Numbers in parentheses denote the number of animals at each point.

Glucagon induction of the enzyme also is initially inhibited by actinomycin D, but later in the induction the antibiotic has no effect, suggesting some action of the hormone at the translational level. Corticosteroids induce serine dehydrase relatively inefficiently and only after a considerable lag period (14). Furthermore, if animals are maintained on a protein-depleted diet, virtually no induction of serine dehydrase by corticosteroids is possible (15); however, glucagon still induces the enzyme to quite high levels. Glucagon induction of the enzymes, ornithine transaminase and histidase, also occurs, whereas (like serine dehydrase) these enzymes are relatively unaffected by corticosteroids. On the other hand, glucagon administration has little effect on tryptophan pyrrolase or tyrosine transaminase. Thus it would appear that different gluconeogenic hormones affect different classes of liver enzymes.

In view of the finding of glucose repression, as well as nicotinamide repression and tryptophan stabilization, it is apparent that the control of genetic expression in mammalian liver may occur at the level of the mRNA template. In all cases this presupposes the stability of a mRNA template, a phenomenon which may be relatively rare in microorganisms. As seen from text-figure 1, the sustained translator, which is the stable template, may be controlled by various environmental conditions. The experiments outlined here support this concept which may indicate a major difference between the control of genetic expression in mammalian tissues as opposed to microorganisms.

CONTROL MECHANISMS EXPRESSED *IN VITRO* COMPARED WITH THOSE *IN VIVO*

In a recent review (16) Eagle considered a number of examples of the environmental and genetic control of enzyme levels, morphology, and specific functions of mammalian cells cultured *in vitro*. We will not attempt to expand on this compendium, but rather will consider certain specific examples in some detail, with special emphasis on the biochemical expressions of related cells *in vivo* compared with those *in vitro*.

In his review, Eagle considered some of the important factors in the initial explanations of cells from an *in vivo* to an *in vitro* environment. The primary changes with which the cell is faced in its *in vitro* environment are a dilution of metabolites, essential and nonessential, a possible alteration in macromolecular environment such as horse serum for human plasma, etc., and a drastic decrease in population density. The decrease in population density may lead to certain changes in nutritional requirements which were not readily apparent when the tissue was growing *in vivo* (16). Also, during this initial period of growth *in vitro*, dramatic enzyme changes may occur in the cellular population such as the loss of

ornithine transcarbamylase in explanted liver as described by Sato and co-workers (17). Lieberman and associates (18, 19) have studied the alterations in RNA and DNA synthesis occurring in rabbit kidney cortex cells during the initial growth of these cells in a new *in vitro* environment. How this is related, if at all, to the compensatory hypertrophy that occurs *in vivo* in the remaining kidney after removal of the other is not clear. Furthermore, although hepatic regeneration may be stimulated dramatically *in vivo* by removal of 70% of the liver, explantation of adult liver cells to culture has not yet resulted in successful growth, although it is possible to maintain liver cells without growth (17). Fugimoto and Harary (20) reported that rat heart cells before explantation to culture have a respiratory quotient of 0.81 indicating a high level of fat metabolism, whereas cells cultured for a week show a considerably higher respiratory quotient, close to 1.0, indicating a shift from fat to carbohydrate metabolism. Obviously there are many possible mechanisms that cause these changes, not the least significant of which is the selection of a minority cell type during the culture period *in vitro*. As we shall see later, the only relatively foolproof methods of insuring that the population has remained stable are by a maintenance of its enzymology or by reinoculation into a suitable host animal with evidence of reestablishment of the original tissue *in vivo*.

The control of biochemical expression in primary cultures has not been widely investigated. Lieberman and associates (21) have described a substrate-induced increase in hexokinase activity in kidney cells explanted directly from *in vivo* to *in vitro* conditions. Sixty-five hours after explantation in the presence of glucose, hexokinase levels are 3 times higher than cultures without glucose. Changes in lactate dehydrogenase, glucose-6-phosphate dehydrogenase, adenosine deaminase, and malate dehydrogenase are relatively insignificant during the same time period. An interesting example of the maintenance of certain enzymatic functions upon explantation by manipulation of the environment has been described by Twarog and Larson (22) who reported that bovine mammary cell cultures *in vitro* lost the ability to produce lactose unless the lactose was added to the culture medium. Using a fluorescence microscopy technique, Granick (23) was able to demonstrate an increased δ -amino levulinic acid synthetase activity in liver parenchymal cells when cultured in the presence of chemicals capable of inducing acute porphyria. Actinomycin and mitomycin added to the culture prevented the increased enzyme activity as did puromycin or parafluorophenylalanine. The induction of this enzyme in liver has been demonstrated (24), and Tschudy and associates (25) have shown that the disease of humans, acute intermittent porphyria, may be the result of an uncontrolled synthesis of this enzyme.

Somewhat more impressive, although perhaps not quite so well defined, are examples of the control of biochemical expression during initial ex-

plant growth which may be seen during embryonic differentiation *in vitro*. Rutter *et al.* (26) have demonstrated the growth and differentiation of pancreatic epithelium grown *in vitro* for 1 week. During this period there was a dramatic increase in the development of zymogen granules coincidental with a dramatic increase in the activity of pancreatic amylase. In addition, these authors observed that, if actinomycin D were added to the culture initially, no differentiation or enzyme increase occurred. However, if the addition of actinomycin were delayed until 3 days after explantation, then the antibiotic had no effect on further differentiation. These data strongly suggest that once differentiation in this system has occurred, further RNA synthesis is unnecessary and any control of amylase synthesis must occur at the translational level. More recently, Reif and Amos (27) reported that the appearance of the enzyme, glutamotransferase, in the chick embryo retina *in vitro* could be stimulated by some dialyzable factor present in serum. The increase of this enzyme seen in embryonic retina also becomes resistant to the action of actinomycin after a short period (28). It is of interest that earlier DeMars (29) found that this enzyme could be derepressed by the replacement of glutamate for glutamine in the culture medium.

BIOCHEMICAL EXPRESSION OF CELL LINES *IN VIVO* AND *IN VITRO*

In the preceding discussion the control of biochemical expression during the initial stages of cultivation of cells *in vitro* as compared with their *in vivo* progenitors was considered. It is known and will be considered in other portions of this Symposium that continued cultivation of cells in many instances leads to heteroploidy, selection, and other changes in the cellular population, ultimately giving rise to cell strains or cell lines. A major difficulty in any attempt at extrapolation of conclusions derived from these lines is the question of which cell type *in vivo* should the *in vitro* cell line be compared with. In Eagle's review (16) he considers some of the contrasting properties of diploid and heteroploid human cells cultured *in vitro*. In particular, the heteroploid cells appear to have a greater ability to synthesize the amino acid, cysteine, as well as an increased alkaline phosphatase activity. A number of control mechanisms have been considered and studied in established cell lines, many examples of which are considered by Eagle in his review. The phenomenon of repression which, with the exception of glucose repression mentioned previously, is almost nonexistent *in vivo* has been described several times in established cultures. Rickenberg (30) has described an apparent repression of pyrro-

line carboxylic acid reductase in L cells by the addition of proline to the medium. Ennis and Lubin (31) reported that pyrimidine starvation stimulated a threefold to fourfold increase in the level of aspartic transcarbamylase in Sarcoma 180 cells in culture. Schimke (32) also reported that arginine, when added to cultures of cell lines growing in limiting amounts of arginine, causes a drop in the levels of argininosuccinate synthetase and argininosuccinase. The levels of these enzymes in cultures growing in limiting arginine may vary up to 15-fold higher than in those cultures growing in excess arginine. The derepression of glutamine synthetase has already been referred to (29).

As in the studies of enzyme increases *in vivo*, the role of enzyme stabilization or action at the translational level in the control of biochemical expression *in vitro* must also be considered. The work of Schimke (33) indicated that both arginine and manganese, when added to the medium of a growing cell line, stimulated increased levels of arginase, probably by inhibiting enzyme breakdown rather than increasing its synthesis (see text-fig. 1). Schimke (34) did a related study in rat liver and arrived at the same conclusion, indicating a similar mechanism operative *in vivo* and *in vitro*.

IN VIVO AND IN VITRO EXPRESSIONS OF CELL LINES DERIVED FROM RAT HEPATOMAS

As indicated earlier, it becomes very difficult to compare the biochemical expression of cultured cell lines with their actual *in vivo* counterparts. One method of circumventing these problems may be seen in the study of a malignant neoplasm *in vivo* and *in vitro*. Table 2 gives the activity of

TABLE 2.—Enzyme levels of Dunning (D1S1) and Novikoff (N1S1) hepatomas growing *in vivo* and *in vitro**

	Deoxycty- dylc deaminase	TPNH-cyto- chrome-c reductase	Glutamate dehydrogenase	Choline oxidase
<i>In vivo</i>				
D1S1.....	0	102 ± 31	205 ± 65†	74 ± 28†
N1S1.....	300 ± 15	40 ± 2	0	0
<i>In vitro</i>				
D1S1.....	0	200 ± 26	423 ± 126†	160 ± 46†
N1S1.....	320 ± 100	146 ± 52	0	0

*All values, expressed as μ moles product/g net weight/hr., are the averages of 3 experiments $\pm$ standard error of the mean.

†These values are from another line of Dunning, the D129. The D1S1 line had no measurable glutamate dehydrogenase or choline oxidase. (Data from Pitot and Swim, unpublished.)

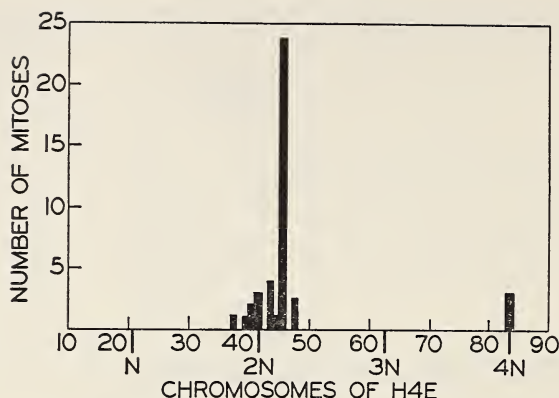
TABLE 3.—Tumors obtained from various inocula of D1S1 and N1S1 cell lines in rats*

	Number of cells in inoculum	Cell lines	
		D1S1	N1S1
1.....	3.5×10^5	1/3	3/3
2.....	1.1×10^5	1/3	3/3
3.....	3.4×10^4	2/3	3/3
4.....	1.1×10^4	3/4	2/4
5.....	3.3×10^3	3/4	3/4
6.....	1.1×10^3	1/4	2/4

*The results are expressed as the ratio of animals with tumor at the time of autopsy to the total number of animals receiving each inoculum. (Data taken from Pitot and Swim, unpublished.)

4 enzymes, deoxycytidylic acid deaminase, TPNH-cytochrome-c-reductase, glutamate dehydrogenase, and choline oxidase, in the Dunning and Novikoff hepatomas grown *in vivo* and *in vitro* (35). As can be seen, there is a relatively close qualitative correlation of these four enzymes *in vivo* and *in vitro*. That this is probably not due to a selection of cells in the different environments may be seen from table 3 in which the results of the size of the inocula on the incidence of neoplasms produced by the two different cell lines may be seen. As few as 1,100 cells inoculated into a homologous strain of animal gave tumors within 4 weeks. These neoplasms were quite heteroploid and probably consisted of several chromosomal lines. Each tumor when inoculated from culture had a rather characteristic histologic pattern. Thus, in this particular case, it appears that the cell growing *in vitro* was probably of the same cell type that grew *in vivo* in the animal.

In the Novikoff and Dunning hepatomas, however, there was no readily available opportunity to study the effect of the environment on the biochemical expression of the cell. Earlier studies by Pitot *et al.* (35) with the highly differentiated Reuber Hepatoma H-35 both *in vivo* and *in vitro* showed that this neoplasm was capable of a high degree of biochemical differentiation while being maintained *in vitro*. The *in vitro* cells were slightly hyperdiploid, exhibiting several extra-small chromosomes (text-fig. 3). At this time this neoplasm exhibited measurable activities of at least a half dozen enzymes found in normal liver as well as in the tumor growing *in vivo* (35). The most striking finding was that the cultured cells growing *in vitro* had enzyme levels comparable to those of the tumor growing *in vivo* in the adrenalectomized animal (table 4). Cortisone administered to the adrenalectomized animal *in vivo* stimulated a substantial increase in the level of this enzyme in the tumor as well as the liver, and data at that time (35) also showed that cortisone acetate added to the culture *in vitro* produced a dramatic rise in the enzyme's level within 6 hours after addition of the hormone to the culture. Thus the tumor grow-



TEXT-FIGURE 3.—The distribution of karyotypes in cultures of the cell line H-4-II-E (data taken from Westphal and Pitot, unpublished).

ing *in vitro* appeared to act, at least with respect to this enzyme, in a manner very similar to the tumor growing *in vivo* in the adrenalectomized animal.

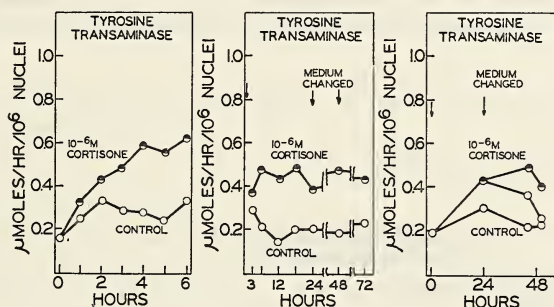
More recent studies of this neoplasm *in vitro* designated as the H-4-II-E cell line which has now been maintained in continuous culture for more than 4 years show that the heteroploidy has increased to a mode of 102. Intraperitoneal inoculation of the cells back into the animal produces tumors, but these neoplasms now grow quite rapidly destroying the host in as short a time as 2 weeks and have a more anaplastic appearance histologically than the original tumor (35). However, the response of the H-4-II-E to cortisone is still present as evidenced by the recent data of Potter *et al.* given in text-figure 4 (36). In these separate charts the kinetics of the increase of the activity of tyrosine transaminase *in vitro* in the presence of 10^{-6} M cortisone acetate is noted. When the medium is changed and cortisone removed, the level of the enzyme comes back down to the control level. Studies by Morse (37) have shown that addition of

TABLE 4.—Tyrosine α -ketoglutarate transaminase activities of liver, Reuber H-35 hepatoma, and the cell line H4-II-E*

	Liver	H-35	H4-II-E
Control <i>in vivo</i>	15-50	200-600	
+ cortisone <i>in vivo</i>	220-300	300	
Adrenalectomized <i>in vivo</i>	20-55	50-80	
+ cortisone <i>in vivo</i>	120-450	130-300	
Control <i>in vitro</i>			80-200
+ cortisone <i>in vitro</i>			400-680

* See reference (35) for details.

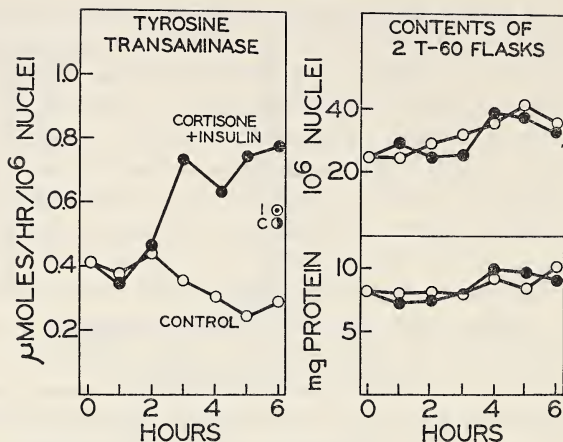
actinomycin D or puromycin to the culture along with the cortisone acetate results in a complete inhibition of the increase in the enzyme level. Recent work of Thompson *et al.* (38), using another cell line derived from a rat hepatoma in which tyrosine transaminase may be induced by steroids *in vitro*, has shown that when actinomycin is added to cultures after the enzyme is fully induced, a further increase in the level of the enzyme is noted. The increase in nuclei and protein of the culture during the first 6 hours is seen in text-figure 5. Also shown is the enhancement of the cortisone effect by insulin. To date, no studies of the administration of both cortisone and insulin to tumor-bearing adrenalectomized animals have been done. In order to demonstrate that the increase in tyrosine transaminase in the presence of cortisone, plus or minus insulin, is not a general phenomenon, the activity of the enzyme, ornithine transaminase, in H-4-II-E cells is plotted on a bar graph (text-fig. 6). The very stable and constitutive level of ornithine transaminase is noted in this graph. Thus it appears that even after more than 4 years in culture the selective hormonal control of tyrosine transaminase is still present in this cell population.



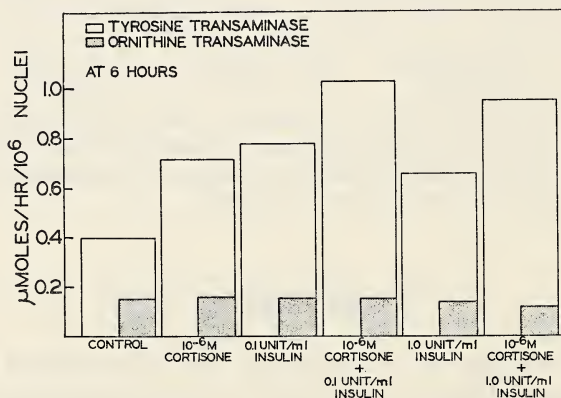
TEXT-FIGURE 4.—Effect of cortisone on tyrosine transaminase activity of cultures of the cell line H-4-II-E. Each point denotes the average of at least three determinations. See reference (35) for details of culture and enzyme assay methodology. Karyotype mode is 102. [Data of Potter *et al.* (36).]

CONCLUSIONS

From this brief survey, it is apparent that cells cultured *in vitro* are capable of several different mechanisms for the environmental control of biochemical or genetic expression. In fact, in the case of enzyme repression more examples are known *in vitro* than are known *in vivo*. Furthermore, the "differentiated" state of cells even after extended culture *in vitro* may be again exhibited during *in vivo* growth and the enzymology of certain neoplasms may be indistinguishable in the *in vivo* or *in vitro* state. These may be special cases, but on the other hand such systems may offer promis-



TEXT-FIGURE 5.—Effect of addition of cortisone and insulin on the levels of tyrosine transaminase in cultures of the H-4-II-E and the change in protein content and nuclei number of cultures during the first 6 hours of enzyme induction. The single points designated I and C indicate the 6-hour levels of enzyme in cultures given either insulin alone (I) or cortisone alone (C). The level of insulin in the culture media was 1 unit per ml. *See reference (35) for further details. [Data of Potter et al. (36).]*



TEXT-FIGURE 6.—Levels of tyrosine transaminase and ornithine transaminase in cultures of the cell line H-4-II-E under various cultural conditions. *See legends of text-figures 4 and 5 for further details. Each bar represents the enzyme level at a time 6 hours after addition of the hormones. [Data of Potter et al. (36).]*

ing tools in the study of the mechanisms involved in the transition from *in vivo* to *in vitro* environments. The problem of cell selection during this transition is obvious and may only be overcome by conditions which favor differentiation in the resting state rather than proliferation (16, 17). As yet the artificial environments of tissue culture are decidedly not those of the "internal milieu," but with the continued advance of our knowledge, as exemplified by this Conference, the day when the artificial may equal the natural is not far off.

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DISCUSSION

Dr. Hsu: Thank you, Dr. Pitot. I think all of you would agree with me that this was a lucid presentation of a wonderful subject. I would like to call Dr. Robert Schimke to discuss this subject. Then we will open it to the floor.

Dr. Schimke: First of all, I congratulate Dr. Pitot on some very elegant studies. He mentioned some of my own work. I perhaps will mention this only in passing.

I will direct my comments to the more general problem occurring, as I see it, in animal tissues. This has to do with what it means to have genetic expression. I bring this up as an occupational problem, an experimental problem, in terms of how one determines at what level and under what circumstances the expression or what happens when one affects a system of a cell culture or an animal tissue acting at the genetic level, *i.e.*, by affecting the rate of a messenger RNA synthesis. Ideally, this could be done by studying the product of this reaction—in other words, the messenger RNA. This is, of course, a very big problem, and there are two potential methods now available. One would be to find and isolate specific messenger RNAs. One method being used in all cases is sucrose gradient. I think you will all agree this is a very poor method which does not allow a fractionation of the specific RNAs. Another method which might be of value in the next 10 years—I hope before the next review by this society—involves the hybridization techniques. Now, at the moment, they are very crude, but I think, in time, this may be a very valuable tool.

If we cannot measure the specific RNA, then we must measure other products of gene expression. We have to measure the end products, proteins, or enzymes.

Now here is where we really get into an involved problem. Dr. Pitot and previous speakers have mentioned that in animal tissues there may be a number of levels at which the rate of synthesis of a specific protein may be controlled. I will mention a few of the known levels because we sometimes tend to think of the control as operating at only one level.

Currently it is held that in *Escherichia coli* the genetic expression occurs at the level of the synthesis of mRNA. However, when we examine yeast and animal tissues, far greater complications ensue. I infer that it is probably the major theme of this meeting to inquire into the complicated nature of the process of genetic expression.

We mention translational control, but this is a nebulous term. Transitional control could mean control that occurs either at the level of where this messenger is attached to the ribosomes or at the level where the specific transfer RNAs are attached in the process of assembling the peptide.

Another limiting control could be the rate of release from the ribosome of the assembled peptide. The latter hypothetical control has been proposed as occurring in hemoglobin synthesis or as operating in ferritin synthesis. To say this in a different fashion, you have on the ribosome a subunit not released, but when another subunit

reacts with it or another ligand, such as a metal, the peptide is taken off the ribosome site, leaving the site free for synthesis of a new peptide.

Dr. Pitot alluded to another problem with which I have been much concerned. This is that the amount of enzyme in an extract of animal tissue at any given time depends not only on the rate of synthesis of the protein but also on how rapidly it is being broken down. It is appropriate to ask: Can findings in studies such as these be used as criteria of genetic expression? I think in general they probably can. This is made necessarily so. But I would like to insert a note of caution. In unpublished work of Saminsky with yeast (obviously not mammalian cells, but neither simple bacteria) as the source of several malic dehydrogenases, he found that, if he added magnesium ion to the medium under growing conditions, he obtained an enzyme that he could purify and characterize. This "magnesium" enzyme had a certain amino acid sequence; but if he used cobalt instead of the magnesium he got a dehydrogenase with very similar properties, but a different amino acid sequence. It is pertinent to ask: Are these metals affecting the genetic expression? Saminsky thinks not. He thinks that the metal acts at the ribosomal site to effect the release from it of one or another of two different types of peptide. He postulates that this must occur at the translational, not the genetic level. I bring up these findings because in a rigorous proof of action at the genetic level one must always question where the given agent is working.

To conclude these remarks, I would like to say that perhaps this is the end of any simplistic approach to the genetic, or otherwise, control of protein synthesis, certainly in animal tissues and also probably in bacteria. We must realize this is an extremely complex problem, and I think that cell culture and the solution to the problems of maintaining differentiated cells in culture are going to go a long way toward understanding this complexity.

There are several questions that perhaps will not be covered that I think ought to be raised here.

One is the relationship of the cell cycle, that is, the replication of the chromosome to the synthesis of specific proteins. This is known to occur in *Bacillus subtilis* and in yeast. Recently, Dr. Littlefield described this as also occurring in cell culture of thymidylate kinase. I wonder if this is a general phenomenon in cell culture or in all cells.

I would like to raise another question. When one studies protein synthesis and uses this as the end stage of genetic expression in the broken cell, one is dealing with, at most, 5% of the rate of synthesis of protein as it is observed in the intact cell. So I wonder what has happened to that other 95% and what this has to do with the destruction of the cellular structure which is undoubtedly extremely important not only in control of protein synthesis but in the entire process.

In addition, I would like to raise one technical point related to Dr. Pitot's presentation. This has to do again with the problem of interpreting an enzyme level in relation to the fact that the enzyme is being both synthesized and broken down. This is probably true of all the enzymes that have been discussed here. We have found that the inactivation or breakdown of enzymes is extremely sensitive to all sorts of procedures. These procedures include the administration of puromycin and actinomycin D. In fact, with tyrosine transaminase, the decay of the enzyme, which is more dramatic in the intact animal than perhaps it is in the cell culture, is entirely prevented by agents that inhibit protein synthesis and also partially inhibited by actinomycin D. This is also true of tryptophan pyrrolase. It is another enzyme that turns over very rapidly.

So again, one must be cautious in the use of these drugs, for they may have more effects than one originally thinks.

Dr. Hsu: Thank you, Dr. Schimke. Before we open this discussion period to the floor, would Dr. Pitot like to make some comment?

Dr. Pitot: I think we are all fully aware that the use of such drugs presents many problems. The work of Dr. Schimke and other investigators performing similar studies certainly has prompted us to do the immunochemical studies reported here. Before these experiments were undertaken, however, we did try some other conditions besides actinomycin D to test these phenomena, particularly in respect to the template lifetime studies.

Working with Dr. Sam Leshner at the Argonne Laboratories, we were able to show that γ -radiation has an actinomycin-like effect on the enzyme serine dehydrase. For example, one can delineate a period of enzyme synthesis which is insensitive to γ -radiation, this time being very close in length to that of actinomycin-D resistance. Furthermore, one may also use the antimetabolite fluoroorotic acid in similar studies. Heidelberger and Wagner (Biochim Biophys Acta 61: 373, 1962) have shown this compound to be incorporated into liver RNA as a uridine moiety.

By administering this compound during enzyme induction, one also gets a period of fluoroorotic resistance of enzyme induction like that of actinomycin.

Furthermore, from preliminary data using fluoroorotic acid *in vivo*, Dr. Jost of our laboratory has shown that after the initial administration of casein hydrolysate the animal is able to produce a protein which is immunochemically very similar to serine dehydrase, but which has absolutely no enzymatic activity, suggesting that incorporation of fluorouracil into RNA results in the production of a false message, as has been done in several microbial systems.

Dr. Harary: I would like to comment on the so-called paradoxical effect of the actinomycin and puromycin. We must be aware of the possibility that there are differential effects of actinomycin and puromycin on protein synthesis. For instance, one might inhibit more severely the synthesis of the other enzymes or other proteins that one is looking at.

We have studied malic dehydrogenase and some other enzymes in cultured heart cells and have seen an increase in specific activity resulting from the administration of puromycin.

Yet the total enzyme concentration goes down. This indicates that puromycin affects the synthesis of the other enzymes or proteins more effectively than the synthesis of the enzyme we are looking at, such as the malic dehydrogenase. Therefore, there is an increased specific activity but a decreased total activity of the malic dehydrogenase.

Dr. Pitot: I have never seen the kind of action you describe with puromycin. But certainly knowledge of the paradoxical effect of actinomycin has been with us for some time, although it has been interpreted in different ways. For example, Reich and Goldberg, in a review of actinomycin effects some 2 years ago (Progr Nucleic Acid Res and Molecular Biol 3: 183, 1964), mentioned the phenomenon, which is well known today, that RNA viruses grow much better in tissue cultures in the presence of actinomycin than in its absence. Since RNA virus replication is relatively resistant, if not completely resistant, to the antibiotic, one might classify this as a paradoxical effect of actinomycin where the mechanism is understood.

On this basis, we have considered the paradoxical effect of actinomycin as a shutting-off of all RNA synthesis, including what we referred to as "brief translator" synthesis and expression. Such an effect would allow all of those amino acids and other metabolites that would normally be utilized for the expression of "brief translators" to be used for the enhanced expression of stable templates.

Dr. Hsu: Dr. Schimke, would you like to comment?

Dr. Schimke: One can mimic the so-called paradoxical effect with tryptophan pyrrolase by amino acid administration and also simply with tryptophan. Inasmuch as actinomycin D does increase the free amino acid level in the liver to a slight extent, I think there is a question as to what level it is worth it.

Dr. Hsu: Yes.

Dr. Harary: I think my point relates more to your point, Dr. Schimke, about the turnover of these enzymes. If you consider that malic dehydrogenase, for example, is a stable enzyme which turns over very slowly, it could perhaps persist in a system where the other enzymes do not persist and where the level of those enzymes might depend upon *de novo* synthesis. In that case, you will get the so-called paradoxical effect.

Dr. Schimke: Right. Do you know whether you actually had a decrease in the amount of total mass of tissue in your experiments?

Dr. Harary: Yes. Not decrease in total mass, but decrease in the extractable protein.

Dr. Schimke: Then, the explanation you give could be very correct.

Dr. Stone: We have studied cells originating from the Chinese hamster hypothalamus. With respect to Dr. Schimke's remarks, we have found that on losing a group-3 chromosome, we lose activity of monamine oxidase. And in synchronized cell cultures by the double thymidine block, monamine oxidase increases in the operon.

Dr. Katsuta: Many cancer researchers in Japan have developed and established lines of transference of prostatic tumors of rats. They examined the cell properties of these lines and found tremendous variation.

I am gratified to hear this lecture by Dr. Pitot. Before comparing the normal tissue and the laboratory-grown tumor, as well as the slow-growing hepatoma cells, we must compare and rank the variation in properties of slow-growing hepatoma cells. Otherwise, some extreme cancers taken from one case might be taken for the general phenomenon of slow-growing tumor cells.

Dr. Pitot: If I understood the comment correctly, I think the point is that one should consider slow-growing rather than fast-growing hepatoma cells. We agree fully.

Certainly, some of the tumors that Dr. Morris has produced are extremely slow-growing. For example, one that I did not mention here, the Hepatoma 7787, takes almost 1 year to kill the animal. Yet mechanisms controlling genetic expression are equally defective in this neoplasm as in some hepatomas that cause the death of the host in less than a month. All the tumors exhibit extensive defects in control mechanisms, although the defects are relatively unique for each neoplasm.

This certainly leads to a fantastic mosaic of differences. But we feel, by merely looking at the mechanisms controlling enzyme levels and enzyme synthesis, that these are defective in all neoplasms, no matter what their growth rate.

Dr. Prehn: In the so-called humorous text-figure 1 with which the lecture was opened and closed, I noticed that the stable template was a little spiral, and the unstable one, a sine wave. My question is whether this is the humorous or serious part of the slide.

Dr. Pitot: When I first made up this text-figure, I considered this difference seriously, feeling that the spiral-type polysomes (cochleosomes) were found only in association with membranes. This led to something else we had been discussing for some time, that the sustained translator may in most cases be the polysome associated with membranes.

Since then, I found this concept is a mistaken one. There are free polysomes not in apparent association with membranes, which are just as spiral as those that are bound. So you are right, it may be the humorous portion of the text-figure.

Dr. Jacoby: Did Dr. Pitot say that, in the hepatoma cells in culture *in vitro*, there was a complete absence of synthesis of glucose-6-phosphatase?

Dr. Pitot: Glucose-6-phosphate dehydrogenase.

Dr. Jacoby: Was there part absence of this or complete lack of it? Has an attempt been made to demonstrate it histochemically in the hepatoma *in vivo* in order to decide whether it was in the stroma or in the actual hepatoma cells?

Dr. Pitot: No.

Dr. Lostroh: Dr. Pitot, do you have any evidence as to where glucose may act to repress the synthesis of the serine dehydrase? Secondly, what substrates other than glucose can cause this repression?

Dr. Pitot: We have been interested in the mechanism of the glucose repression and have been studying it for some time. Our ideas about the mechanism have very recently undergone a drastic change, although the experimental data at the present are only suggestive. Initially, we found a correlation between the deposition of glycogen in the liver and the presence of glucose repression. This was first indicated by the fact that glycogenolytic hormones, *e.g.*, glucagon or epinephrine, will relieve the glucose repression effect. That is, if one administers glucose followed by glucagon to the animal, complete relief of the glucose repression is obtained.

This argues that glucose itself is probably not the effector. We went on, then, to suggest that perhaps glycogen, which is usually found in association with the smooth endoplasmic reticulum, was sterically interfering with the functioning polysome. Since that time, we have found one glaring exception to this model. Cortisone, when given to an animal, stimulates a considerable deposition of glycogen in the animal, yet it does not effectively repress enzyme synthesis. However, in the last few months Dr. Jost has been investigating polysome binding, using immunochemical mechanisms. It appears now that one effect of glucose repression is to prevent the release of the finished polypeptide chain from the template.

To answer your second question, fructose works just as well as glucose. We have tried several other sugars, but usually they are fairly toxic to the animal.

Dr. Amos: I just want to make one comment on the question and to expand Dr. Harary's statement that the loss of protein in cells is perhaps fairly significant. If you do just DNA-to-protein ratios under some of the conditions of stoppage of protein synthesis, you can conclude that the cells are practically being destroyed and in fact being lost. So I am not sure that Dr. Pitot answered the question of whether the total synthesis or the total amount of enzyme really changes or if you always do specific activities, because if you do specific activities, you may face that problem.

Secondly, if valine is made by the liver, how do you account for the fact that glucose also reduces the amount of total incorporation by 40% in insoluble proteins? It may not be statistically significant, but the discrepancy between the amount of enzymatic activity in the glucose repressant in 6 hours is still quite out of line with the incorporation of 850 down to 150. Then the enzyme has been cut in half. It looks almost as though you have a modification in these cells. Maybe they make valine from glucose.

Dr. Pitot: The valine given here is in pulse amounts, less than a microgram.

Concerning your other question, we feel that—and I think Dr. Schimke has emphasized this point—the level of the enzyme may not reflect the rate of synthesis. This is certainly true with respect to the delayed glucose repression.

As shown in the text-figure, we were able to induce the level of serine dehydrase to a certain point and then abruptly shut off any further increase with glucose. But the enzyme itself stayed at this higher than base level. Yet the rate of synthesis of the enzyme as measured by the immunochemical technique was essentially that of the zero time control. Thus the actual level of the enzyme does not necessarily reflect its rate of synthesis.

Dr. Littlefield: I am confused about the use of the words "induction" and "repression." For example, thymidine kinase that we have studied is often referred to as an inducible or repressible enzyme, yet our data indicated that it really wasn't, but that it was constitutive, if anything, with its levels entirely governed by the amount of substrate stabilizing it. So I would add emphasis to that aspect of dealing with these activities.

Dr. Wilde: Dr. Pitot, did you study the enzymes of the glycolytic cycle under conditions of glucose repression in amniotic embryos? We have been studying either heterobiosis or cyanide drops or uridylyluridine incorporation of RNA down to zero—completely. Is there a link?

Dr. Pitot: Glucose administration has no effect on the incorporation of orotic acid into rapidly labeled nuclear RNA, nor does it have any effect, as the text-figure shows, on general protein synthesis as measured by ^{14}C -valine incorporation into protein.

The only glycolytic enzymes we have investigated under the conditions of glucose repression are hexokinase and glucokinase. Glucose repression is obtained only when conditions favor the induction of the enzyme glucokinase. Glucagon relieves the glucose repression and also inhibits the induction of glucokinase by glucose.

Dr. Amos: Dr. Littlefield's remark is important. I, too, am misled by the term "repression." I think you are talking about the level of the genetic repression.

You replied to my question by saying the destruction of the enzyme, the stability, perhaps was more the clue and not necessarily synthesis. But then, if you insist on calling this repression, it does make it look as though we are getting into a semantic problem that may be a very important one.

Dr. Pitot: I agree entirely. I am using the terms "induction" and "repression" only to indicate an increase or decrease in the rate of synthesis of the enzyme. Generally these terms are used here in the same sense as in microbial systems, although a certain genetic mechanism is implied. No genetic mechanism is implied here. By this definition, we are saying it is possible to have enzyme repression on a stable template, *i.e.*, while the system is insensitive to actinomycin D. The reason the enzyme remains high but the rate of synthesis is virtually zero or at least at basal levels is that the enzyme is stabilized by the amino acids present, preventing its degradation. In fact, if you do not give amino acids along with the glucose, the enzyme level decays.

Dr. Schimke: I agree with this. This is a battle I have been fighting for a number of years—the use of the words "induction" and "repression" as they apply to animal tissues. I wish we could think of some more nondescript terms that don't imply microbe-type mechanisms.

Genetic Markers as Tracers in Cell Culture^{1, 2}

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SUMMARY

This paper deals with the use of various levels of genic expression to distinguish between cell cultures of different origin. DNA replication patterns, variations in the types of RNA molecules synthesized by different tissues, and the persistence of certain genetic markers in cell culture are considered. It is suggested that DNA-RNA hybridization as a means of comparing tissue and cell culture RNA molecules offers a promising tool for answers to questions of tissue origin-cell culture relationships. A survey of certain genetic markers in established human cell lines

revealed the possibility of a serious problem of intraspecific contamination. Eighteen established human cell lines of supposed independent origin were shown to be identical with regard to their glucose-6-phosphate dehydrogenase (G6PD) (A) and phosphoglucomutase (1) types. Since G6PD A type has been found only in Negroes, and HeLa is from a Negro and was the first established human cell line, it is concluded that these 18 human cell lines are most likely HeLa cell contaminants.—*Nat Cancer Inst Monogr* 26: 167–195, 1967.

THE HOPE that cells in culture would exhibit the metabolic and immunologic characteristics of the tissue from which they were derived has met generally with disappointment. Except for neoplasms to be discussed later, cell cultures from one tissue are much the same as cell cultures from another within the same organism. I will address myself to the question of whether levels of genic expression can be used to distinguish between cell cultures of different origin. Since other speakers will discuss this question at the protein-enzyme level later in the Conference, I will confine myself to certain other aspects of genetic expression, such as variations in DNA replication patterns exhibited by mammalian chromosomes, variations in the types of RNA molecules synthesized by cells of

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different origin, and the persistence of certain genetic markers in cell culture.

The use of genetic tracers in cell culture has revealed a technical problem which can be serious in certain types of investigation, namely, the problem of intraspecific contamination. Interspecific contamination of laboratory cultures is of course a well-known hazard; such contamination can be readily detected, however, by immunologic and cytologic techniques (1-3). Intraspecific contamination is more difficult to detect and has been largely ignored. We have recently acquired evidence showing that most, if not all, permanent heteroploid human cell lines are contaminants. This report will include information on the use of stable genetic markers as tracers to assure cell line purity.

RELATIONSHIP OF CELL CULTURE TO TISSUE OF ORIGIN

Variation in Karyotype

The concept is generally accepted that the karyotype, except for ploidy, is essentially constant through development in mammalian forms. A variety of observations including chemical (4), cytophotometric (5), and cytologic investigations support this view. The recent work of McCarthy and Hoyer (6) comparing DNA from different mouse tissues as well as "L" cell DNA by the DNA-DNA hybridization technique is especially significant. Their analysis indicated identity of DNA sequence in all tissues studied (brain, kidney, thymus, spleen, and liver) and also in L cell DNA. In view of the long *in vitro* history of the L cell and its many chromosomal rearrangements, this latter finding suggests a strong selective pressure for maintenance of the total genome. These *in vivo* observations are supported by chromosome studies on *in vitro* cultures derived from a number of different tissues, karyotype identity being the rule (7).

Although there is little karyotypic variation, and therefore the karyotype has not been a useful character for the analysis of relationship between cell cultures and their tissues of origin, there are instances, not sufficiently investigated, where the karyotype might be diagnostic. Liver tissue, for example, has been found to have polyploid cells by chemical and cytophotometric methods. The occurrence of polyploid lines from liver tissue would therefore provide evidence of origin. In the creeping vole (8), the X chromosome varies between gonadal and somatic tissues. Perhaps further study will reveal other persistent differences that can be used to establish origin.

Chromosomal variation results also from meiotic and early mitotic errors. The aberrant karyotypes thus produced are tissue-wide in their distribution. As culture markers for genetic investigations and for cell identification, these aberrations have been useful in a variety of studies.

Some of these abnormal karyotypic variations are tissue-specific and could be of value in the analysis of the relationship between tissue of origin and the derived cell culture. The cell chimerism and associated chromosomal mosaicism in opposite-sexed cattle twins are restricted to the hematopoietic system and could be used to determine whether cell cultures derived from bone marrow biopsies represent bone marrow parenchyma. Similarly, in chronic myelogenous leukemia, one of the G chromosomes is replaced by an abnormally small chromosome (the Philadelphia), the abnormality being found only in hematopoietic cells. Although the condition is mosaic, it could also be used in the study of cultures derived from bone marrow.

Chromosome Replication Patterns

The best studied and documented replication patterns involve the sex chromosomes where the well-known late replication patterns of one of the X's (chromatin body forming X or inactive X) and the Y have been intensively and extensively studied (9-25). These studies have been carried out on bone marrow, leukocytes, spermatogonial cells, and cell cultures derived from various tissues. Except for spermatogonial cells [a single study on the Chinese hamster (15)], the basic asynchrony of sex chromosome replication (one early X and one late X in females and an early X and late Y in males) appears to be a mammalian characteristic with tissue-wide distribution.

The sex chromosome replication patterns are maintained under a variety of conditions, including numerical and structural abnormalities (12, 17-19) and even in established cell lines (9, 19). The situation in bovine cells is especially instructive, since here the X and Y can be distinguished from the autosomes and each other without any ambiguity (13). In female cells, typical asynchronous labeling of the X chromosome is exhibited. In XY cells the Y chromosome is the last to initiate DNA synthesis and one of the last to complete it. The Y chromosome retains its replication pattern even when it is translocated to another chromosome. This has been shown in an established cell line (MDBK) started from a bull calf, in which the Y chromosome appears to be translocated to a large autosomal chromosome. In a clone of this line derived by Nelson-Rees (24), the marker chromosome with the Y-replicating element attached is no longer present and a cytologically Y-like chromosome can be identified in many cells. This Y-like element again replicates like the Y in normal cells (figs. 1 and 2).

The general permanence of sex chromosome replication patterns under a variety of conditions and in different tissues is not unexpected in view of the relationship of the asynchrony of sex chromosome replication to dosage compensation. If late replication in autosomes reflects genetic inactivity, as it apparently does in sex chromosomes, conceivably the

autosomal distribution of late replication may vary characteristically in different tissues. That is, each tissue may have a particular set of inactive autosomal genes and, providing they are sufficiently clustered, characteristic localized late replication patterns could result. In man, skin-derived cell cultures and short-term leukocyte systems have been extensively used in chromosome replication studies. Although the emphasis in these studies has been mainly the sex chromosomes, the autosomes have not been neglected, and, to my knowledge, no striking or consistent differences between tissues in autosomal replication patterns have been reported. Hsu (16), Utakoji and Hsu (15), and Pflueger and Yunis (25) compared chromosomal replication patterns of a number of tissues in the Chinese hamster and found no significant differences between tissues. More subtle intertissue replication differences may well exist, but the absence of detectable ones with present methods of resolution indicates that DNA replication patterns are not likely to be useful as tracers for study of cell culture-tissue derivation problems.

Tissue-Specific Protein and RNA Populations

It appears well established that tissue-specific phenotypes are not retained in normal adult mammalian cell cultures, regardless of the origin of the cultures. On the other hand, phenotypes with a tissue-wide distribution, *e.g.*, glucose-6-phosphate dehydrogenase (G6PD), are detectable at the cell culture level, and, as might be expected, genetic differences affecting such characters are also measurable *in vitro*.

In contrast to this general picture for normal cell cultures, there is considerable evidence for the retention of tissue-specific characters in cultures derived from tumors. A culture derived from a mammalian mast cell tumor produced 5-hydroxytryptamine and histamine (26), whereas cultures derived from pituitary and adrenal tumors produced adrenocorticotropin and steroids, respectively (27). Recently Pitot *et al.* (28) have derived a culture from a rat liver hepatoma which has retained activity for a number of liver enzymes. Retention of tissue-specific characters has also been well demonstrated in chicken embryonic cultures from muscle (myotube formation) (29), retina (pigment production) (30), and cartilage (cartilage production) (31).

Several hypotheses have been proposed to explain the loss of specialized function in culture. The selection hypothesis states that the parenchymal cells present in an organ or tissue fail to grow, or grow slowly and are eventually completely overgrown by a nonfunctional cell type present as a minority population in the tissue of origin. The differentiation hypothesis states that the conditions of *in vitro* cell culture are unfavorable to specialized function and cause the cells to revert to an undifferentiated state.

The immunological and enzymatic study of Sato, Zaroff, and Mills (32) on cultures derived from rat liver was one of the first careful analyses of

this question. Their immunological tests involved the reaction between cell cultures derived from liver and rabbit antisera prepared against various rat tissues. These experiments showed that cell cultures lacked liver-specific antigens but had antigens found in cell cultures derived from other tissues and in *in vivo* nonhepatic tissues. On the basis of these results, they concluded that only the nonparenchymal cells of the liver grew in culture, thus favoring the selection hypothesis. This study would appear to be a model in the use of tissue-specific antisera for determining the relationship of cell cultures to their tissue of origin.

It is also possible that cell cultures retain their tissue-specific properties, but at a level of protein synthesis too low for detection by the usual methods. More sensitive techniques, such as the use of fluorogenic substrates (33-35) for enzyme assays, might reveal a low basal rate of synthesis. Also, cell cultures may retain tissue-specific RNA synthesis, even though they no longer produce the corresponding proteins. The DNA-RNA hybridization technique of McCarthy and Hoyer (6) is extremely powerful in detecting very small differences in the types of RNA molecules being synthesized. Distinct RNA populations from mouse *in vivo* lung, kidney and spleen, and *in vitro* L cells were demonstrated by these workers. By the same method, they were able to show that a mouse embryonic primary kidney culture continued to synthesize an RNA population similar to its *in vivo* counterpart. Obviously this same method can be applied to the study of different cell lines and strains derived from various tissues and maintained for varying periods in culture; such studies should provide information at the RNA level on tissue-cell culture relationships.

PERSISTENCE OF KNOWN GENETIC VARIANTS IN CELL CULTURE

Table 1 lists the known *in vivo* markers that have been identified at the cell culture level. As indicated earlier, these markers represent gene products, tissue-wide in distribution. Rare variants such as galactosemia and orotic aciduria are useful as tags for individual cell strains and various genetic studies. Polymorphic variants like G6PD and phosphoglucomutase (PGM) variation also have value as markers for *in vitro* genetic studies and are especially useful in the detection of intraspecific cell culture contamination.

The problem of interspecific cell culture contamination is already an old one. Several instances of mouse L cell contamination of supposed human cultures and those of other species have been clearly demonstrated by the use of such characters as karyotype, sensitivity to polio virus, and various immunologic procedures (1-3). In intraspecific contamination these same cytologic and immunologic systems are of little value. Poly-

TABLE 1.—*In vivo* genetic variants detectable in human cell cultures

Variant	Reference
Acatalasia (Japanese variant).....	36
Acatalasia (Swiss variant).....	37
Glucose-6-phosphate dehydrogenase (G6PD) changes:	
Electrophoretic variation.....	38
Mediterranean variant.....	39
Familial nonspherocytic hemolytic anemia.....	40
Galactosemia.....	41
Maple syrup urine disease (deficiency branched chain amino acid oxidative decarboxylase activity).....	42, 43
Orotic aciduria.....	44
Phosphoglucomutase (PGM) electrophoretic variation.....	45
6-Phosphogluconic dehydrogenase (6PGD) electrophoretic variation.....	45
Lactic dehydrogenase electrophoretic variation.....	45
Hurler's disease.....	46
Adenylic kinase (AK) electrophoretic variation.....	47

morphisms detectable in cell cultures are, on the other hand, useful, their identification capacity increasing according to the power of the number of genetically independent systems available. Also, the more equal the allele frequencies for any polymorphic system the more useful it becomes. The situation is identical to the use of the blood group and serum protein polymorphisms in paternity testing and in the determination of zygosity in twinning. Unfortunately, the blood group and serum protein variants are not detectable in cell culture and at present only four polymorphic systems are available for cell culture work.

These four systems all involve electrophoretic variants of different enzymes: G6PD, 6-phosphogluconic dehydrogenase (6PGD), PGM, and adenylic kinase (AK), and more than 200 phenotypic combinations of them can be distinguished electrophoretically. However, the frequencies and distribution of these polymorphisms are such that on the average the probability of two independently derived cultures having the same phenotypes for all four systems is quite high, being over 25%. With increasing numbers of polymorphisms, the detection of intraspecific cell culture contamination should become a technical formality.

In certain situations even the presently available systems can be very useful, and recent work of ours involving the G6PD system in human established cell lines will illustrate this point. The major observations involve the G6PD zymogram patterns of 18 human cell lines reported to be of independent origin (table 2). They all represent the well-known heteroploid, epithelial, permanent cell type, and in most instances their history suggests an origin by spontaneous transformation from non-epithelial cells. The G6PD variants that concern us are the A (fast) and B (slow) types (fig. 3) (48, 49). The A type has been found only in Negroes and is composed of two subtypes (A⁺ and A⁻) not separable by starch gel electrophoresis. The combined gene frequency of the two alleles

(G6PD^{A+} and G6PD^{A-}) controlling the A band is approximately 30% in the American Negro population.

The results of our G6PD analyses of these supposedly 18 independently derived human cell lines are that all have the A band. Dr. Yoshida of our department has developed a chromatographic system, modified from Luzzatto and Allan (50), for separating the A⁺ and A⁻ G6PD variants. He has kindly assayed four of these lines (HeLa, Detroit 98, AV₃, and WISH) and finds them all to be A⁺ (the frequency of the A⁺ allele in American Negroes is about 20%).

I have not been able to ascertain the supposed racial origin of all 18 lines; it is known, however, that at least some of these are from Caucasians (*e.g.*, KB and WISH), and that at least one, HeLa, is from a Negro (51). Thus a statistically based sampling explanation of these results is excluded. A further argument against a statistical explanation comes from the results of our PGM starch-gel electrophoretic analysis which showed all 18 lines to have the identical PGM type 1 (fig. 4), a variant with an approximately 65% frequency in both Negro and Caucasian populations (52).

Three explanations for these observations present themselves for consideration: 1) G6PD expression is modified in human established cell lines so that only the A band is found; 2) A type cells are selected as the precursors of established cell lines; or 3) all these lines are HeLa cell contaminants. The first argument is countered by the following facts: SV40 cellular transformation does not alter the cellular G6PD phenotype. We have examined three cell strains infected with SV40 and, in all, the G6PD electrophoretic type was B. We have also examined one hypodiploid line (53) and one quasi-diploid line (54, 55) derived from tumors just a

TABLE 2.—Established heteroploid human cell lines analyzed in this study

Original designation and tissue source	Supply for this study*
HeLa (carcinoma, cervix)	I (four lines assayed)
Detroit-6 (sternal marrow)	C
Minnesota-EE (esophageal epithelium)	I
Intestine 407 (embryonic intestine)	C
Chang liver (liver)	I; ATCC
KB (carcinoma, oral)	C
Detroit-98 (sternal marrow)	ATCC
NCTC 2544 (skin, epithelium)	ATCC
AV ₃ (amnion)	ATCC
HEp-2 (carcinoma larynx)	I
J-111 (monocytic leukemia)	C
WISH (amnion)	I; ATCC
Giradi heart (heart)	ATCC
HEK (human embryonic kidney)	C
Heart	I
Leukocytes	I
Leukocytes	I
Embryonic	I

*I = individual investigator; C = commercial source; ATCC = American Type Culture Collection (53).

few years ago and still maintained in continuous culture. Both these lines have the B electrophoretic band. Our recent demonstration of only single phenotype leiomyomas in G6PD A/B heterozygotes (both A and B tumors being found in each) is also pertinent (56). Due to the cell mosaic nature of G6PD heterozygotes, tumors arising from single cells in such heterozygotes should exhibit one or the other of the hemizygous phenotypes, and as indicated, they do. If modification of G6PD expression in abnormal growths was the rule, then we should not have found both A and B tumors in these cases.

The second argument requires a heterogeneous population from which A type cells may be selected. G6PD A/B heterozygotes are such populations and, as we have indicated (57), selection does occur *in vitro* in such systems and may even occur *in vivo*. When cell cultures are started from G6PD heterozygotes, early transfers have always shown the presence of both kinds of cells; however, one of the cell types eventually takes over the culture and in most instances, though not always, the A cell type predominates. This tells us incidentally that a G6PD type A cell line could have been derived from an A/B individual. The tumor work is again relevant, in that if selection of A cells was a significant factor, one would expect an excess of A tumors over B, both in number and size. Neither of these expectations is found. If A type cell lines developed from Caucasians (homozygous or hemizygous B), the A cells would first have to arise by mutation. The existence of permanent B type near-diploid tumor lines (53-55) argues against the efficacy of the mutation-selection route as the explanation of A type established lines from B type individuals.

It seems to me the simplest explanation of our observations, that the established heteroploid human cell lines are all G6PD A, is that they are all HeLa cell contaminants. The lines were examined before cultivation in my laboratory; thus contamination preexisted. The fact that the HeLa line is from a Negro and was the first developed human cell line also supports the argument of contamination. Practically all the reported established human cell lines (at least those listed by the American Type Culture Collection) were derived within 5 years of HeLa (1952-57) and very few have been reported since. The termination of this period coincides with the appearance of the first report on the commonness of interspecific cell culture contamination (1). Following this first report and others on interspecific contamination (2, 3) there are virtually none of so-called spontaneous transformed human cell cultures; this suggests a sudden awareness of technical errors and the introduction of corrective measures.

Unfortunately, cell culture contamination, both interspecific and intraspecific, will continue to occur, especially in the more inexperienced laboratories. A number of lines given to me as recently transformed human cultures turned out to be cases of interspecific contamination. In this respect I wish to point out that starch gel electrophoresis of a single enzyme, PGM,

can serve not only as a partial check on intraspecific contamination but as a very powerful test for interspecific contamination (fig. 5). Thus far every species we have checked (man, hamster, rat, rabbit, mouse, and cow) has a distinctive PGM zymogram, and the entire analysis takes less than one day's work of a single technician for up to 20 samples.

The acceptance of the conclusion that most, if not all, the heteroploid, epithelial human cell lines are HeLa cell derivatives poses some important questions. For one thing, it points up the remarkable stability of normal human cultures, that is, the virtual absence of spontaneous cellular transformation among them. It would seem from our work that the only established human cell lines, except for viral transformed cultures, have been derived directly from tumors. Cellular transformation will be the subject of a later session at this Symposium and I will not consider it any further. Of more immediate concern are questions regarding the significance of past work with these various cell lines. In instances where the investigator's requirement is a human cell line, and where any would do, the problem is not important. However, in those cases where specific characteristics of the cell line are involved, it is important to know whether the characteristics were acquired *in vitro* or supposedly reflected the *in vivo* tissue origin of the line. Thus, where the investigator has assumed a specific tissue of origin of the cell line, *i.e.*, liver, leukocytes, or bone marrow, the work is open to serious question, and in my opinion would be best discarded. In the future, the incorporation of stable genetic markers in material to be cultured is the best guarantee against contamination.

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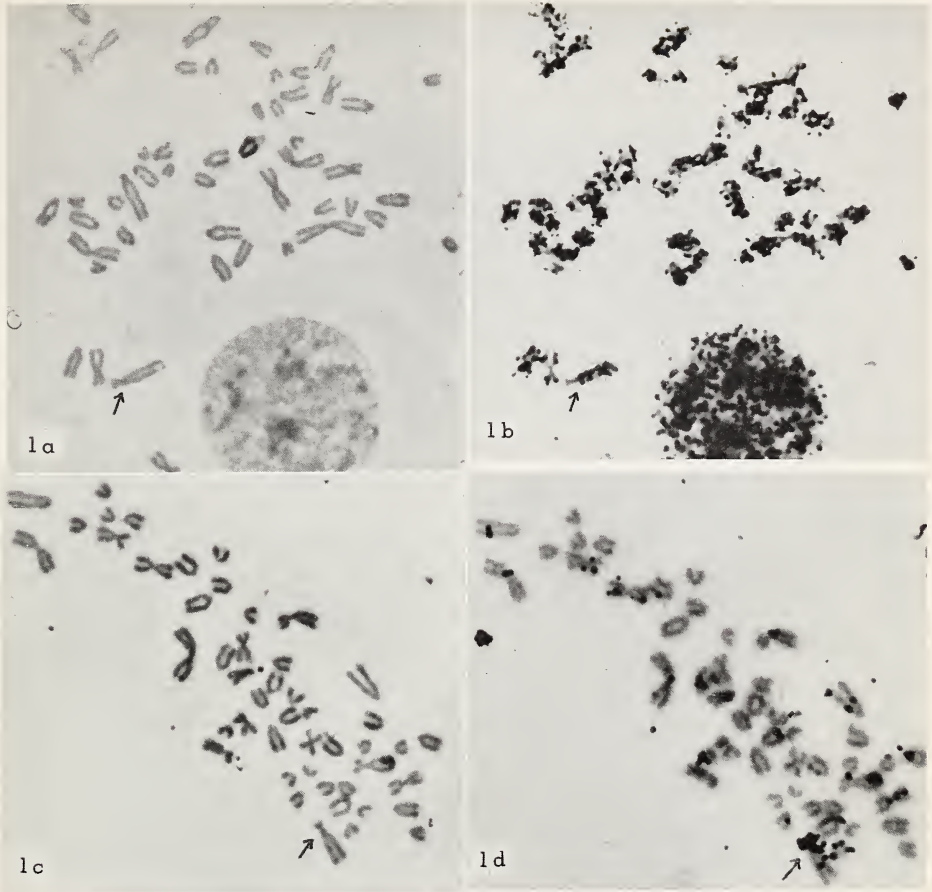


FIGURE 1.—Prephotograph and postphotograph of radioautographed cells of MDBK; *a* and *b*: precells and postcells from an early part of the S period; *c* and *d*: precells and postcells from the late part of S. *Arrows* mark Y-carrying chromosome.

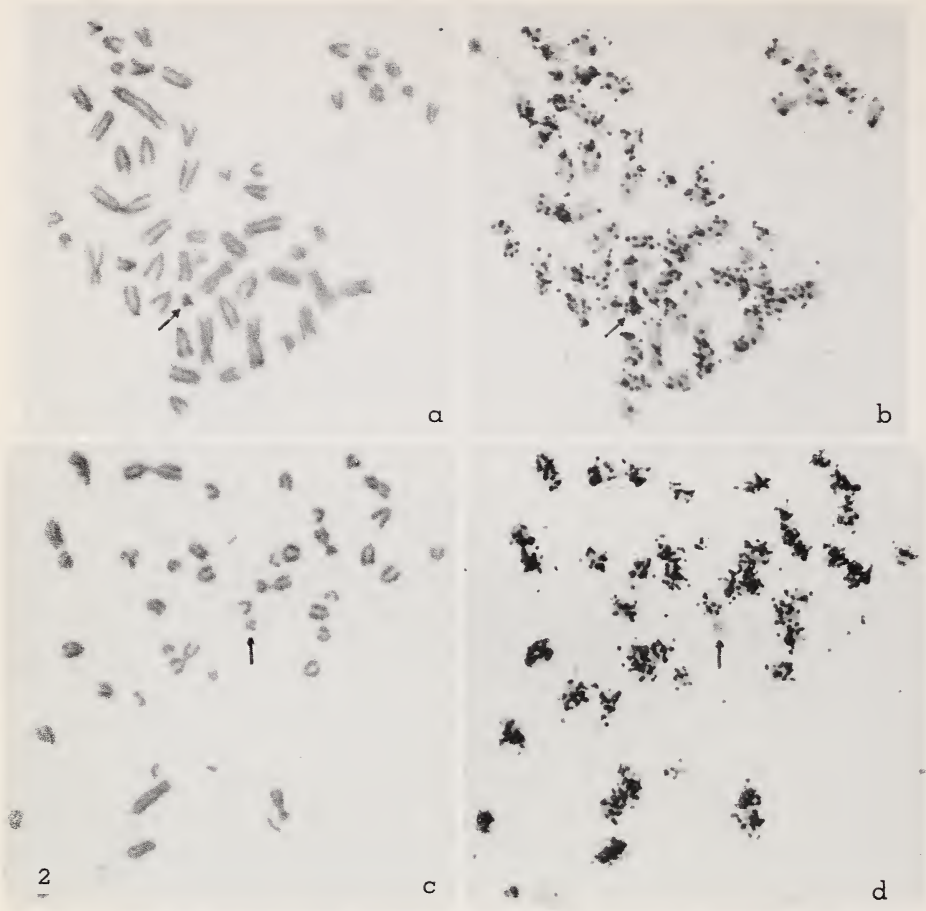


FIGURE 2.—Prephotographs and postphotographs of radioautographed cells of MDBK (clone 958-9; Nelson-Rees); *a* and *b*: precells and postcells from the late part of the S period; *c* and *d*: precells and postcells from the early part of S. Arrows mark presumed Y.

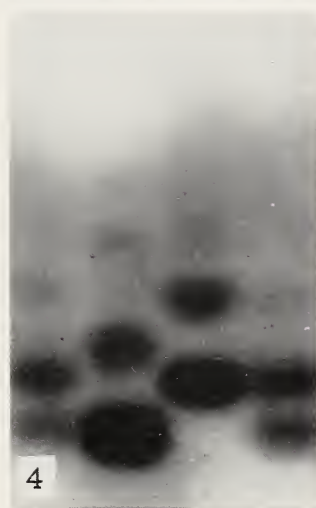
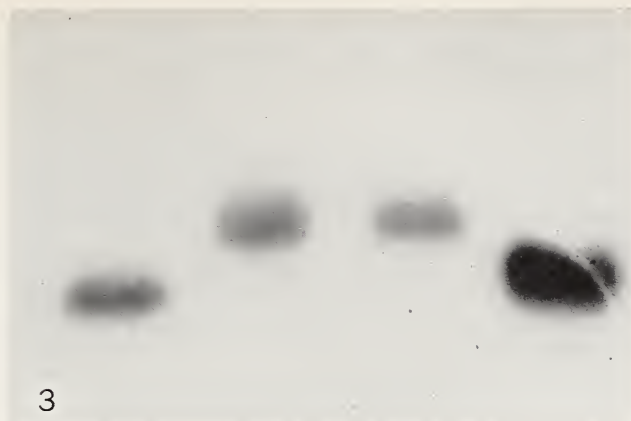


FIGURE 3.—G6PD electrophoresis of human cell culture extracts. Slots from *left to right*: 1, slow (B) ; 2, fast (A) ; 3, fast (A) ; and 4, slow (B).

FIGURE 4.—PGM electrophoresis of human cell culture extracts slots from *left to right*: first, 1-2 ; second, 1 ; third, 2 ; and fourth, 1-2.

FIGURE 5.—PGM electrophoresis of Chinese hamster, rat and man. Slots from *left to right*: 1, Chinese hamster ; 2, rat ; and 3, human.

DISCUSSION

Dr. Hsu: A few years ago I voiced some suspicion about cell-line contamination, after the monumental work by Rothfels *et al.*, but there was no way to tell, differentiate, or identify the human cell line, since immunological techniques would fail. So I am happy about the paper by Dr. Gartler and am also sure he has made many people unhappy. I would like to ask Dr. Margery Shaw for a discussion.

Dr. Shaw: Dr. Gartler has very ably reviewed the genetic variants that are presently detectable in human cell cultures and has made the plea that "... stable genetic markers in material to be cultured is the best guarantee against contamination." I would like to add that stable genetic markers will be not only useful but necessary for somatic cell genetics of the future, and it would behoove us all to begin a collection of them for free exchange in the scientific community. To the list of 11 biochemical markers in Dr. Gartler's table may be added at least one more: the hexose-6-phosphate dehydrogenase (hexose-6 PD) autosomal polymorphism, reported only last week in *Science* independently by my husband C. R. Shaw¹ and by Ohno *et al.*² The patterns have been shown to be stable in both liver and kidney cells derived from fresh human autopsy material, and this new isozymic system is demonstrable in HeLa cells,³ which should be of interest to Dr. Gartler.

The present rate of discovery of electrophoretic markers is steadily increasing, although not all enzymes present in fresh tissues are produced by cells cultured from those tissues. We have almost come to expect a monthly announcement of a new multiple-allele or multiple-locus polymorphic system from the Galton Laboratory of Professor Harry Harris, in London, who is with us here.

We are just beginning to realize the extent to which enzymes vary in natural populations. For example, Lewontin and Hubby⁴ recently found that approximately 40% of the hydrolase and dehydrogenase loci tested in wild populations of *Drosophila* exhibit electrophoretic polymorphism. Since MacCluer has calculated that approximately 28% of all single amino acid substitutions are electrophoretically distinguishable,⁵ the outlook is certainly optimistic. When we have better methods for detecting "silent" amino acid and single nucleotide substitutions, it would be surprising if our enzymic individuality is not as unique as our fingerprints!

Dr. Gartler has emphasized the need for genetic markers to monitor human cell cultures for possible contamination. There are many other projected uses of these markers: cell hybridization experiments, evidence for somatic crossing-over, complementation, and cell selection *in vitro*, to mention just a few.

I would like to discuss another marker system which has been useful in the past and will be even more so in the future: chromosome markers. Human chromosomal polymorphism is much more widespread than was expected even a few years ago. These "markers" may be classified as structural variants and numerical variants, both of which have been shown to be remarkably stable in human fibroblast cultures.

Figure 1 shows the karyotype of an infant with the *cri-du-chat* syndrome. The "marker" here is a chromosome in the B group (autosomal pairs 4 and 5) in which part of the short arm of one chromosome is deleted. Since this chromosome is morphologically dissimilar to any other chromosome in the human diploid comple-

¹ SHAW, C. R.: Glucose-6-phosphate dehydrogenase: homologous molecules in deer, mouse and man. *Science* 153: 1013-1015, 1966.

² OHNO, S., PAYNE, H. W., MORRISON, M., and BEUTLER, E.: Hexose-6-phosphate dehydrogenase found in human liver. *Science* 153: 1015-1016, 1966.

³ SHAW, C. R.: Personal communication.

⁴ LEWONTIN, R. C., and HUBBY, J. L.: A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics* 54: 595-609, 1966.

⁵ MACCLUER, J. W.: Quoted in Shaw, C. R.: Electrophoretic variation in enzymes. *Science* 149: 936-943, 1965.

ment, it is readily distinguishable and useful as a marker. Many other chromosome structural variants are easily obtained in the human population by screening both normal and abnormal individuals. A few examples of these are listed below:

A. X chromosome

1. Long arm deletion
2. Isochromosome (long arm)
3. Ring-X chromosome
4. Centric fragment

B. Y chromosome

- *1. Unusually long Y
- *2. Metacentric Y (? long arm deletion)
- *3. Pericentric inversion

C. Acrocentric chromosomes (group D and G autosomes)

- *1. Deletion of satellites and heterochromatic stalk
- *2. Elongated short arms (? duplication)
- *3. Tandem satellites
- *4. Giant satellites

D. Other autosomal variants

1. *Cri-du-chat* chromosome (*see* fig. 1)
2. Philadelphia chromosome (deletion in long arm of #21)
3. Short-arm deletion of #18
4. Long-arm deletion of #18
5. Autosomal rings
6. Reciprocal translocations
- *7. Exaggerated secondary constriction region of #1, 9, or 16

*Minor variants not associated with abnormal phenotypes.

With our present methods, the frequency of human chromosome structural variation (including the "minor" variants) has been estimated to be as high as 5%. With improved methods in the future, such as measurement of the DNA content of single chromosomes and computer analysis of karyotypes, this figure will undoubtedly be higher.

If chromosome structural markers are not required, and numerical variants can fill the needs of the experimenter, an ample supply of numerically marked cell cultures is now available. It has long been known that human amnion cells are easily maintained *in vitro*, and these are now a ready source of heteroploid tissue.

Dr. Carr has shown that, among 200 spontaneous human abortions, fetal and amniotic tissue in culture showed a numerical chromosomal abnormality in 44 cases.⁶ These included XO, triploids, tetraploids, and trisomies for all of the autosome groups except group F. His findings have been corroborated by at least 5 other series, and it is now estimated that the frequency of heteroploidy in embryos aborted in the first trimester of pregnancy is higher than 25%. So if you want a cell strain with 47 chromosomes you need only go to the nearest individual with Down's syndrome or the nearest obstetrician.

Since Dr. Gartler has discussed the use of electrophoretic markers in identifying the origin of cultured human heteroploid cells, I would like to suggest another possible use of these enzyme variants: mapping human autosomal genes by utilizing cell cultures derived from trisomic individuals.

⁶ CARR, D. H.: Chromosome studies in spontaneous abortions. *Obstet Gynec* 26: 308-326, 1965.

ONE LOCUS - TWO ALLELES							
	DIPLOID			TRISOMIC			
<i>Genotype</i>	<i>AA</i>	<i>AB</i>	<i>BB</i>	<i>AAA</i>	<i>AAB</i>	<i>ABB</i>	<i>BBB</i>
<i>Frequency</i>	p^2	$2pq$	q^2	p^3	$3p^2q$	$3pq^2$	q^3
<i>Isozyme Pattern</i> (No hybrid band)							
<i>Isozyme Pattern</i> (Hybrid band)							

TEXT-FIGURE 1.—Theoretical isozyme patterns produced in homozygotes and heterozygotes of diploid and trisomic populations. If the enzyme occurs as a monomer, no hybrids bands appear (*top*). If random association of allelic subunits occurs, hybrid bands appear (*bottom*). In the diploid heterozygote (*AB*) the grayness of the bands represents a 1:1 ratio and 1:2:1 ratio, respectively, in staining intensity. In the trisomic heterozygotes, *AAB* and *ABB*, the ratios are 2:1, 4:4:1, 1:2, and 1:4:4, respectively.

Text-figure 1 diagrams a hypothetical example of an isozyme pattern that is polymorphic in the normal human diploid population. Here there are 3 genotypes: *AA*, *AB*, and *BB*. Now let us assume we have a trisomic culture in which the genetic locus for the enzyme involved is on the chromosome that is present in triplicate. Now we have 4 genotypes: *AAA*, *AAB*, *ABB*, and *BBB*. If all 3 chromosomes contribute equally to the isozyme product, then we might expect a shift in the quantity of enzymes produced in the trisomic heterozygote. For example, in the *AAB* individual, the 2 bands are of unequal staining intensity. The *AA* band would be expected to be twice as dark as the *B* band.

Many enzymes are multimeric, *i.e.*, composed of two or more polypeptide subunits, and the most common form is the dimer. In this case, and if the subunits associate randomly, a hybrid enzyme is formed in the heterozygote, and it migrates electrophoretically midway between the two homogeneous dimers. If hybrid enzymes are formed (as shown in the lower half of text-fig. 1), the middle hybrid band is usually twice as dark as the other two in the diploid state, giving a 1:2:1 ratio in staining intensity. Furthermore, we might again expect, in the trisomic heterozygote, a shift in the relative quantities of the isozymes. Here we predict 3 bands in the *AAB* heterozygote, with a relative theoretical staining intensity of 4:4:1, if the dimers are formed from randomly associating subunits.

Text-figure 2 shows how the pattern is predicted in a 3-allele system in a diploid population. This is not just an idle exercise, since Hopkinson, Spencer, and Harris⁷ have demonstrated 3 alleles in the human red cell acid phosphatases, and Shaw and Barto⁸ have shown 4 alleles in the hexose-6-PD polymorphism in

⁷ HOPKINSON, D. A., SPENCER, N., and HARRIS, H.: Genetical studies on human red cell acid phosphatase. *Amer J Human Genet* 16: 141-154, 1964.

⁸ SHAW, C. R., and BARTO, E.: A multiallelic system of glucose-6-phosphate dehydrogenase variation in *Peromyscus maniculatus*. *Genetics* 52: 474, 1965.

the deer mouse. Again we have the hybrid band pattern in a 1:2:1 ratio in the diploid heterozygotes, AB , BC , and AC .

As shown in text-figure 3 in the trisomic population there is a shift in the staining intensity in the first 6 heterozygotes (AAB , ABB , BBC , BCC , AAO , and ACC). However, a unique isozyme pattern emerges in the ABC phenotype: 5 bands instead of 3, in the ratio of 1:2:3:2:1.

With cell culture techniques already available, and with the anticipated abundant supply of enzyme and chromosomal markers, we can hope to witness the beginnings of human autosomal genetic maps in the near future. Perhaps at the next Decennial Review Conference we will have established several linkage groups and calculated map distances between a few loci.

Dr. Hsu: I hope the American Type Culture Collection representatives, Dr. Shannon, and others will heed the plea and store some of these marker strains for future experiments.

Now I would like to ask Dr. Hans Laufer to give us some general conclusions for these somatic cell schematics with insect systems.

Dr. Laufer: A recurrent theme of this Conference seems to be the nature of the controls influencing the expression of genes and the differential readout and control of genetic information during development. I wish to interject a few words of caution by reinforcing and expanding on some of the ideas expressed by previous commentators. What I particularly want to emphasize is that the increase of protein within a cell does not necessarily imply *in situ* or *de novo* synthesis, as Dr. Schimke has demonstrated, even though this appears to be the simplest assumption. Decreased enzyme breakdown or other factors could affect protein concentrations. Proteins synthesized elsewhere can be transported by the blood to other sites. Proteins can accumulate through a decrease in their normal excretion rate from a secretory cell. I want to support the point that Dr. Wilde raised—the continually changing environment in which cells exist. In case of *in vitro* experimen-

ONE LOCUS — THREE ALLELES DIPLOID						
<i>Genotype</i>	AA	AB	BB	BC	CC	AC
<i>Frequency</i>	p^2	$2pq$	q^2	$2qr$	r^2	$2pr$
<i>Isozyme Pattern</i>		  		  		  

TEXT-FIGURE 2.—Theoretical isozyme patterns in a diploid population with enzyme polymorphism due to 3 alleles at one locus. See legend of text-figure 1 for explanation of staining intensity of the bands.

ONE LOCUS - THREE ALLELES TRISOMIC									
AAA	AAB	ABB	BBB	BBC	BCC	CCC	AAC	ACC	ABC
p^3	$3p^2q$	$3pq^2$	q^3	$3q^2r$	$3qr^2$	r^3	$3p^2r$	$3pr^2$	$6pqr$

TEXT-FIGURE 3.—Theoretical isozyme pattern in a population that is trisomic for the chromosome carrying the locus of the enzyme polymorphism shown in text-figure 2. See legend of text-figure 1 for explanation of staining intensity of bands. In the last column, the theoretical ratio is 1:2:3:2:1. (There is an error in the staining pattern of the *BCC* heterozygote. The two black bands should be dark gray.)

tation, it may be possible to achieve such conditions. The environment of cells is continually altered in the osmolarity, changing ion concentrations, and hormone titers during insect development. Finally, I want to consider questions concerning our understanding of differentiation.

First let me introduce some aspects of the structure and chemistry of the dipteran salivary gland. These have giant polytene chromosomes, some of which are $150\ \mu$ long and as much as $15\ \mu$ wide. In *Chironomus thummi*, which we have been using, the chromosomes are completely differentiated into DNA bands and regions active in RNA synthesis, called puffs. The RNA from puffs is presumably messenger RNA. Two or three of the largest puffs, called Balbiani rings, exist in the salivary gland genome. These have been correlated with the process of secretion by a variety of techniques.¹⁻⁴

The activity of these chromosome loci and the development of the salivary gland itself are hormonally controlled. Certain of these puffs, particularly the very large ones, are tissue-specific and are supposedly concerned with the production of the glandular secretion.^{1,3} Here we have a convenient cell system where we can study

¹ LAUFER, H., and NAKASE, Y.: Salivary gland secretion and its relation to chromosomal puffing in the dipteran, *Chironomus thummi*. Proc Nat Acad Sci 53: 511-516, 1965.

² LAUFER, H.: Developmental studies of the dipteran salivary gland. III. Relationships between chromosomal puffing and cellular function during development. In 19th Annual Symposium on Fundamental Cancer Research, M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 1965.

³ BEERMANN, W.: Ein Balbiani-Ring als Locus einer Speicheldrüsenmutation. Chromosoma 12: 1, 1961.

⁴ ———: Control of differentiation at the chromosomal level. Proc of a Symposium sponsored by the New York Heart Association. J Exp Zool 157: 49-61, 1964.

hormonal influences on the activity of the chromosomes as well as the product of this activity.

One can ask whether the secretion is synthesized *de novo* by the gland, implying that the synthesis is directly controlled by chromosomal messengers, or whether there is a more complicated derivation of secretion. The caution I mentioned earlier is related to the possibility of a more complex explanation.

Specific loci of the polytene chromosomes are engaged in differential RNA synthesis and presumably are the sites of messenger synthesis. The loci exist in different tissues and contain specific puffing patterns. These puffing patterns vary from tissue to tissue and we speak of these as tissue-specific. This "tissue specificity" is not the same as that mentioned by Dr. Gartler. I believe he used the term to mean the presence or absence of a particular characteristic or macromolecular species within a tissue. However, tissue specificity as related to puffing refers to the entire puffing pattern. This includes the relative activities of the entire spectrum of loci. This distinction must be kept in mind when one speaks of tissue specificity. For example, in the case of puffing, a locus may be very active in one tissue, and less active or completely inactive in another. This has been observed for several individual loci. Thus, the entire puffing pattern is considered when tissue specificity is spoken of. The activity of loci can be stage-specific as well; that is, their activity may be restricted to special times in the life cycle.

One important question ultimately to be answered is: How do particular chromosomal activities relate to general cellular functions? I don't propose to answer that here for any particular locus, but at least I can indicate the evidence that points to fairly complex interactions.

Is the relationship between chromosomal loci and secretion simple and direct? The simplest hypothesis is that the messenger RNA from puffs controls the synthesis of the protein in the secretion.

Trehalase and hyaluronidase are enzymes in the gland, and they follow a characteristic pattern of increase and decrease during its development. These constituents of the salivary gland are also constituents of the secretion. Their ontogeny seems to reflect the activity of the major puffs, the Balbiani rings. Yet these proteins are not tissue-specific. They exist in other parts of the body, including the blood. Indeed, many blood proteins are also present in the secretion. Injection of radioactive blood proteins results in labeled secretion. Foreign proteins can be injected into the blood of these organisms. In a series of experiments, I¹³¹ human serum albumin was introduced. A considerable amount of this material appeared in the secretion. These constituents are not formed by direct synthesis in the gland, but are accumulated there by being first picked up by it and then transported. This was demonstrated by immunochemical techniques. Thus a major contribution to the salivary secretion is through a transport system.¹ To interpret these data in terms of chromosomal loci, loci should exist that are tissue-specific and not directly involved in synthesis of secretion, rather in the synthesis of a transport system or permeases. These permeases can be viewed as substances which facilitate the picking up and transporting of substances across the gland rather than substances elaborated directly for export by the messengers from chromosomal loci in the gland.

Another instance of an indirect effect of chromosomal activity is the accumulation of yet another enzyme, deoxyribonuclease, in the gland. We found that deoxyribonuclease accumulates by about sevenfold, from one part of the life cycle to another.⁵ This accumulation may be rather significant in terms of development, for it might well account for the subsequent destruction of the salivary gland, which is a larval structure and eliminated during metamorphosis. The enzymatic

⁵ LAUFER, H., and NAKASE, Y.: Developmental studies of the dipteran salivary gland. II. DNase activity in *Chironomus thummi*. J Cell Biol 25: 97-102, 1965.

increase is of special interest, since it precedes by one developmental stage the loss of significant quantities of DNA within the gland. While deoxyribonuclease increases during part of the life cycle, it also constitutes a part of the secretion. As the animal matures, the enzyme decreases in amount and in specific activity from the secretion accumulating within the gland. The increase in deoxyribonuclease within the gland, therefore, is presumably not by means of *de novo* synthesis, but an accumulation of material which had been held back from the secretion. This increase of a particular protein in the salivary tissue then is again not attributable to direct action of known genes, even though, obviously, genes must be responsible in an ultimate, though indirect, way.

Let us return briefly to the problem of the constancy of the environment. The continuous change of the internal milieu of the organism is reflected in the sodium ion concentration in the blood (hemolymph). During the various stages of development, the sodium concentration continually changes within the hemolymph, the blood fluid which bathes the cells of the salivary gland. The sodium concentration, measured by flame photometry, is a continually changing parameter. At the same time, hormonal changes occur. The titer of the juvenile hormone, active during larval stages, presumably decreases in activity just before pupation.⁶ On the other hand, the molting hormone influencing this organism, ecdysone, behaves completely differently. The ecdysone concentration is low in each larval intermolt and increases gradually, coming to a peak with each molt and falling off again thereafter. Considering these factors *in toto*, one can conclude that the environment changes continually within the organism. Can one hope ever to achieve such conditions in culture? Or need one ever achieve these to obtain desired experimental results?

We have been talking about hormone action in several sessions of the Conference. One may ask: Where do these hormones act? Many of you are familiar with the suggestion of Clever and Karlson⁷ that the hormone ecdysone acts directly on the chromosomes. This is an important, yet unanswered, question. The action of ecdysone on puffing, for example, occurs within about 20 minutes after the injection of this hormone, while changes within the cell's membranes occur within 1-5 minutes.⁸ Even though this is rapid, we still know little about the immediate site of hormone action. For example, is there first the formation of a complex between this hormone and a protein, as is suggested for other hormone systems?⁹

Finally, I want to raise an even more critical question which concerns differentiation. We have been talking about changes in proteins within cells and changes in macromolecules in general. These are supposed to reflect differentiation and dedifferentiation of the cell which are ultimately attributable to gene action. Have we changed the differentiation of these cells at all by increasing, decreasing, or even altering their biochemical constitution?

For example, some experiments were done on muscle and cartilage cells.^{10, 11} If one uses the cell's capacity to synthesize a particular macromolecule, are these cells dedifferentiated when they cease to produce their specific product myosin or Chon-

⁶ WILLIAMS, C. M.: The juvenile hormone. III. Its accumulation and storage in the abdomens of certain male moths. *Biol Bull* 124: 355-367, 1963.

⁷ CLEVER, U., and KARLSON, P.: Induktion von Puff-veränderungen in den Speicheldrüsenchromosomen von *Chironomus tentans* durch Ecdyson. *Exp Cell Res* 20: 623-626, 1960.

⁸ KROEGER, H., and LEZZI, M.: Regulation of gene action in insect development. *Ann Rev Entom* 11: 1, 1966.

⁹ GORSKI, J., NOTEBOOM, W., and NICOLETTE, J. A.: Estrogen control of the synthesis of RNA and protein in the uterus. *J Cell Comp Physiol* 66: 91-110, 1965.

¹⁰ HOLTZER, H., ABBOTT, J. L., and HOLTZER, S.: The loss of phenotypic traits by differentiated cells in vitro. I. Dedifferentiation of cartilage cells. *Proc Nat Acad Sci USA* 46: 1533-1542, 1960.

¹¹ HOLTZER, H.: Aspects of chondrogenesis and myogenesis. In *Synthesis of Molecular and Cellular Structure* (Rudnick, D., ed.). New York, Ronald Press, 1965, pp 35-38.

droitin sulfate in culture, or do they retain their capacity to synthesize these molecules, should conditions be changed and become favorable again? Are these differentiated irreversibly in the sense that once having differentiated, they may not do so along alternative pathways?

The question is rather difficult to answer. The experiments by Hadorn¹² on genital discs in *Drosophila* are particularly relevant here. These tissues normally develop into accessory genital structures. However, if they are cultured over a long period, with several transfers after they have been permitted to regenerate following sectioning, other derivatives are occasionally obtained. That is, wing, eye, and antennal structures, which would normally never be derived from such a source, are observed. Thus it seems that the differentiated state of these cells is modified after a long period. I wonder whether the gain or loss in laboratory cultures of one or two molecular species due to changes in the synthetic activities of cells represents this same sort of change in differentiated state. Do other fundamental changes occur when the developmental capacities of cells are altered, as might be suggested by Hadorn's results?

Dr. Harary: I would like to comment on Dr. Laufer's last point about the problem of dedifferentiation and differentiation. We must come to some definition of terms here. I don't propose to do this right now except to point out the possibility of alternate pathways. Alternate differentiation really does not define whether a cell has dedifferentiated. As an example of an exception to this, I refer to the ability to take a fully differentiated carrot cell, put it into culture, and differentiate this into the leaf, stem, and root cells.

One has varied potential in differentiated cells in this process—its ability either to dedifferentiate and differentiate or redifferentiate into other kinds of tissues. I don't know how insects stack up with carrots, and how an insect compares with mammalian tissues, but it is obvious that each species has a different capacity in this regard. One has to be careful about the definition in relation to the species.

Dr. Laufer: I agree.

Dr. White: I want to comment on the hideous suggestion in regard to this paper of Laufer that this sort of capacity to revert to a totally potent cell is by no means limited to the carrot or to plants, but that it also occurs in mammals. I would cite the work of Dr. Stevens in our own laboratory with the teratoma testis of the mouse in which, if one makes a suspension of these cells and introduces them intraperitoneally, every cell will develop into an embryo which goes on to the neural tube stage. They are perfect duplicates of embryos.

These cells from the male mouse have the average capacity to develop into typical embryos. They go on to about the 5-day stage and then, due to pressures, go over into a teratoid type of growth.

Dr. Laufer: There are many examples of this sort, but, again, when we try to determine dedifferentiation, we don't really know what the pathway is. The way some people use the term "dedifferentiation" implies that the same path is used as was used originally to get there. We don't really know anything about this.

Dr. Evans: Dr. Gartler, after you received your cell strains from the American Type Culture Collection or from individuals or, according to Dr. Waymouth, from commercial sources, how long did you keep them in your laboratory?

Dr. Gartler: They were analyzed before being grown in my laboratory.

Dr. Evans: They didn't send them to you frozen?

Dr. Gartler: We can analyze frozen samples. There is no problem.

¹² HADORN, E.: Konstanz, Wechsel und Typus der Determination und Differenzierung in Zellen aus männlichen Genitalanlagen von *Drosophila melanogaster* nach Dauerkultur in vivo. *Develop Biol* 13: 424-507, 1966.

Dr. Stevenson: When you get these cells from individuals or commercial sources, do you check up on the passage history of them and where they originally came from?

Dr. Gartler: No. In certain instances I tried to get the same culture from more than one source. But except for that, I did not make any particular attempt to find out whether a particular culture had been through 100 passages or anything else about its history.

Dr. Stevenson: It has been about 5 years since some of the obvious dangers of carrying more than one heteroploid cell line in a laboratory have been pointed out. Unless you have a very carefully controlled method of production of the cells and the base reference culture, work of this type, although very interesting, certainly cannot be definitive.

Dr. Coriell some years ago showed it was possible to transfer HeLa cells by aerosol contamination. Anyone who carries three or four heteroploid cell lines in a laboratory, without sufficient markers in those cell lines to distinguish them from each other, runs into grave risk of cross-contamination.

One reason for setting up the cell culture collection was to provide a baseline of cultures to go back to, so that one could have some definitive results based on the passage history and identification of the cells.

It looks as if more detective work is needed to see whether these things really are all derivatives of HeLa and whether we are going to have to start all over again to isolate some new heteroploid human cell lines.

Dr. Gartler: Is your point that the cultures I tested, which were obtained from individuals or commercial sources, may have been contaminated in these laboratories?

Dr. Stevenson: Right.

Dr. Gartler: I gather that you would accept only the ones from the American Type Culture Collection as being true representatives of particular cell strains.

Dr. Stevenson: Yes.

Dr. Gartler: Then I am not too sure I understand your point.

Dr. Stevenson: Well, we can only go back so far and define the best possible passage history to the originator of the cell line. If something occurred at the time he originated it, which allowed him some cross-contamination with HeLa, this is a problem. If we don't have markers, we can never tell. However, if on subsequent passage through many hands, the buddy system, giving somebody a cell line that you messed up in your own laboratory, maybe unknowingly, compounds the difficulties. The only way you can get back to it is to return to the originator of the cell lines as we have tried to do.

Dr. Hsu: I would like to give particular priority to those who initiated the cell lines, whom Dr. Gartler has attacked. If there is any defense, we would like to hear it.

Dr. Deinhardt: I have another question for Dr. Gartler. In your table 2, do you know how many of the 17 cell lines you checked were of Caucasian origin and how many might have been of Negro origin?

Secondly, I would like to add a word of caution in the use of karyotypes at the level of observation available to us today in identifying or pinning down specific cell strains or lines. Karyotypes certainly are most valuable in determining the species of origin. On the other hand, however, experiments done during the last 2 years by Miss Troy and Dr. Mashinsky in our laboratory gave some rather astonishing results.

Starting from one kidney of a nonhuman primate, which was cultured several times, going back to liquid-nitrogen material over 2 years and over 100 tissue culture passages, we found these cell strains started out good diploid, that many of them died between 50 and 70 passages, that some others developed typical marker chromosomes such as ring chromosomes, dicentric, or trisomy. These increased in one instance up to 90% of all cells. Other cell strains that have lost contact inhibition, but that still have a normal morphology, are, up to 140 passages, completely diploid.

If I had reported on these cell strains 40 or 50 passages ago, they would have been heteroploidy and no one knows what they will be in another 50 passages.

These studies are so difficult because to karyotype every 5 or 10 passages or to karyotype 30 or 50 cells with a statistical half life, for example, becomes almost impossible if we can't go over to computer karyotyping. If we could, we finally would solve the problem.

We must be cautious not to take karyotype as too serious an identification of a specific line or strain.

Dr. Gartler: As to the racial origin of the strains, all I know is what I stated in my talk; the HeLa cell, according to Dr. Gey, is from a Negro female. The KB strain and WISH are from Caucasians.

I agree with you that karyotype is not useful for cell strain identification.

Dr. Shaw: I agree, too, with the lines which show heteroploidy and many different stem lines. However, many investigators have maintained diploid cell cultures for a number of passages—up to 50—without dicentric markers, ring chromosomes, etc.

Although the monitoring is a little tedious, we routinely monitor 100 cells from each subculture. In Dr. Krooth's laboratory where we are keeping a specific marker such as the *cri-du-chat* chromosome, this can be done very quickly.

I don't think karyotyping is impossible at this stage of the game, but it will be made easier by the computer analysis of karyotypes.

Dr. Hayflick: Dr. Hsu was going to give the highest priority to those listed in Dr. Gartler's table who had developed some of the cell populations. I think I can claim to having developed a cell population to the ultimate degree, having originated it from the ultimate point. But the points I would like to raise, relevant to his thesis that HeLa apparently gave rise to many of the populations listed in the table, are the following:

It seems to me very difficult to accept the idea that all these populations could have arisen from a human contaminant, since populations like L cells have been available for a number of years through this period. One would expect, merely by random distribution, accidents of this type.

Secondly, I am worried about this concept that, since 1957 or whatever the year was, the origin of cell lines has fallen off exponentially. I think this is not true. I think people simply are not reporting these things any more. They have no unique properties, at least no more unique than what we are already aware of. It is tantamount to saying that, since Koch's postulates haven't been fulfilled for things like typhoid and diphtheria for the past decade, the germ theory is in limbo.

I think, as I believe Dr. Stevenson pointed out, it is absolutely essential that all these populations be analyzed for as many diverse sources as possible.

Finally, two short questions: Is it possible to find this fast band "A" type G6PD activity in fibroblasts 100% of the time from material obtained from Negroid donors and, conversely, not from Caucasian donors?

Is it possible that this enzyme activity is capable of being produced by microorganisms? And if so, could this be the origin of some of these calculations?

Dr. Gartler: I believe that my view about the falloff in so-called spontaneous transformations since 1957 will hold up to inspection. I know of no laboratory, where genetic markers are understood and utilized, that has experienced a human cell culture transformation. A very conservative estimate of the number of independent human cultures started from biopsy in these laboratories is well over 1,000.

The answer to your first question is "Yes." Cell cultures from G6PD (A) individuals always exhibit G6PD (A), and Caucasian subjects with G6PD (A) have not been reported.

With regard to your second question, I do not know anything about the G6PD electrophoretic properties of different microorganisms. However, there was no evidence of microbial contamination in any cultures studies. Furthermore, if microbial

contamination was responsible for the fast G6PD band in a culture, then one would expect two G6PD bands, one from the contaminants and one from the culture.

Dr. DeMars: Maybe Dr. Gartler or anyone could help me interpret the results on nucleic acid hybridization that Dr. Gartler referred to. I understand the principle of those experiments that DNA is extracted from differently differentiated tissues and is then mixed in varying proportions with a preparation of RNA. The amount of RNA specifically bound is a measure of the similarity or difference between the two populations of DNA molecules.

The fact that the preparations from L cells and mouse embryonic liver bound similar amounts of RNA is interpreted to mean that those tissues are identical in terms of their genetic information, the DNA.

If those two populations of DNA molecules differed by as little as 1% in their information content, could hybridization techniques, as applied, pick up this difference? One percent in a mammalian genome could mean hundreds of genes, perhaps thousands—plenty to give you different differentiated cells. So what degree of identity can you really detect in this way?

Dr. Gartler: I suspect that one nucleotide difference per hundred cannot be picked up by the hybridization technique. However, possibly one missing gene per hundred or one extra gene per hundred could be detected.

Dr. Chang: Dr. Gartler's paper is very pertinent to taxonomic enzymology. He has presented some interesting data about glucose-6-phosphate dehydrogenase, and he is certainly justified in saying that all the cells he studied have this enzyme and in grouping them on this basis. But it is very difficult to follow his reasoning that, since they all contain this enzyme, they are contaminated.

I also found it difficult to follow some of his other points. He said, "Many of these are cells developed before 1957." There was a time when people became aware that cell contamination occurred. After that time, there was no more publication about the development of the cell. Therefore, he uses this to substantiate his conclusion.

As Dr. Hayflick has pointed out, I don't think people who start transformation of human cells are interested in publishing any more on the development of a cell. We are mainly interested in finding out how we can cause the human cell to transform spontaneously.

We have developed cell lines. For instance, we have developed another line of cells from a human liver biopsy of a total of several liver biopsies we started, but there is nothing to report because it is commonplace.

A recent paper in *Proc Soc* by a group at a medical school reported the establishment of an epithelial-like cell from the normal gum tissue. They have categorically said there is no other cell in the laboratory and, therefore, there cannot be contamination. As Dr. Westfall pointed out, it would be much better, in classifying cells, if we use several attributes instead of just one. This point certainly is very well taken by anyone who has anything to do with that cell.

I will briefly mention a few points about one of the cells in Dr. Gartler's table. 1) He claimed that his cell line was received from an investigator. I don't know that he got it before this meeting; I have never sent him any cell line and I don't remember ever having corresponded with him. He may have gotten it somewhere else. 2) If you would examine carefully the different morphology between the line derived from human liver tissue in the HeLa cell, I think you would agree that the morphology is entirely different. The liver cell line doesn't have the pep and life arrangement of a HeLa cell. Its growth characteristics are entirely different. 3) Interestingly, about half a year ago a publication in the *Journal of Experimental Medicine* by a group at Massachusetts Institute of Technology showed that the liver cell line is about 500 times more resistant to diphtheria toxin than the HeLa cell. (We spent some time looking into it to try to find a lot of our differences.)

Dr. Paul: We are raising a number of questions. One is: What criteria are stable and what are unstable in cultured cells? Before we get onto this general discussion, Dr. German has some remarks to make.

Dr. German: I suggest some possible explanations which might resolve the controversy concerning selection. We have no idea whether there might not be a selection for the genetic type that Dr. Gartler found in each of his cell lines. We know, in aneuploid cell lines, errors in division are common. If one examines mitotic figures, this is obvious. Abnormal, anaphase, and telephase figures are seen. Even after cloning, there is a quick change—a spread around the modal chromosome, indicating nondysjunction or abnormal division of some type. Thus, possibly there is a selection in the culture conditions that have been used in these various laboratories for the phosphoglucosylase type he found. That is assuming that the original line was at least heterozygous at this particular locus.

Selection for that type by our cultural conditions is one explanation for the same type. I don't know whether it is correct. It is a possibility.

Dr. Paul: We have some important questions to consider.

We have recently talked in particular about the possible use of cell lines in genetic studies. This is clearly a subject of great future importance. For geneticists, it is important to have stable markers and cells that are recognizable, preferably at the single cell level or at the clonal level. We must decide whether variation among these clones is due to dimorphisms, environmental influences, or selection, or even contamination. These are real problems which I think we have to try to resolve.

We are ready for questions and comments from the audience. Who would like to begin?

Dr. Hsu: I would like to defend Dr. Gartler. We all remember clearly a number of years ago—maybe 5—when this contamination business began and everybody was very defensive: the L cells contaminating the rabbits, the HeLa cells contaminating the mouse. Those who had the contaminating cells were disturbed. But the evidence on cytological and immunological examination was so overwhelming that very soon this contamination had to be accepted. Now, here comes the HeLa: human contaminating human, whether or not it is HeLa.

I thought the method Dr. Gartler used was interesting and unequivocal, so that the evidence he showed, even though it may not be 100% correct as it stands, is convincing. Certainly we like to think that most, or quite a few, of these strains were contaminants.

Dr. Hirschhorn: Around 1959 at New York University we had a visiting tissue culturist, Dr. Breuner from Zurich, who started, as the only line in his laboratory and as the only tissue culture line of the entire several floors of the school, supposedly a line from human synovial tissue which he then gave to us. At the time, this was the only line in our laboratory. We did some work on it, thought we were working on human tissue, and soon got around to karyotyping it and then testing it immunologically. It was the classic mouse L cell.

Dr. Chang's argument was that he had no other cells, no HeLa cells, around, and this ruled out contamination. I think our experience alone would speak against ruling out contamination. In fact, several of the commercially available preparations, used to fortify media or make up media, go to laboratories in these commercial factories which have lines of this sort running. Although we know that they supposedly sterilize these cultures, these are done relatively openly. And I wouldn't be at all surprised if some of these contaminants didn't really derive from the laboratories in which they were found, but derived perhaps from media used in these laboratories.

Dr. Shannon: I am on the advisory committee of the American Type Culture Collection, and vitally interested in this whole matter. I don't believe that Dr. Gartler or the ATCC or any of the individual investigators should go off the deep end on the basis of these data.

I would like to go on record as saying that the ATCC and the Cell Culture Collection Advisory Committee have long recognized the vital need for the development of means to pinpoint the affinity of cell lines or strains within the human species. We, therefore, would welcome a means to enable us to do this.

While I seriously doubt that all these lines are, in fact, variants of HeLa, I would like to see all the human lines screened by the method suggested by Dr. Gartler, or a similar method. It is rather inconceivable to me, but, of course, possible when one considers all the characteristics of these various cells that are involved.

Dr. Gartler indicated that 5 of the human cell lines in this chart were received from the American Type Culture Collection. There were 13 listed. We actually have 24 or more human cell lines now in the collection.

With respect to some of the characteristics that would not appear to bear this out—for instance, the tumor-producing capacity of the cells—insofar as I am aware, the NCTC 2544 cells (the human skin epithelium) do not produce tumors in the hamster cheek pouch. In the mouse, they would be characteristic of HeLa cells. Instead, they keratinize as one might expect cells of epithelial origin to behave. I believe the same is true of the Detroit-6 cells. Perhaps Dr. Foley, who has more information on this, would make some comments.

Dr. Foley: I can't contribute anything toward the identification of the enzymatic similarity of some of the kernel of the argument. I again point out, however, that all these lines are not alike with respect to their ability to produce tumors in the hamster. In general, the lines discussed were derived from normal tissue. They were not grown in the hamster, whereas the lines derived originally from neoplastic tissue did grow in the hamster.

Dr. Hull: I would like to add just one more parameter to the argument on the identification of these mislabeled cell cultures. At the Detroit meeting on analytical cell culture, we described a method of identifying 25 or 30 cell strains maintained in our laboratory by their differences in susceptibility to a selected group of viruses. Within this group, we had at least 6 or 7 of the human cell strains shown in the table. We noted at that time that we could not prove the strain was, in fact, as the labels indicated, but we can show that each of them is different in susceptibility to these viruses. So, if they are all HeLa, they would represent variants of HeLa in this respect.

Dr. Paul: Thanks, Dr. Hull. I should like to emphasize your statement that they represent variants. I think we should not lose sight of the fact that variants do arise even within clones derived from a single cell—variants in vital susceptibility, variants in transplantability, variants in enzyme activity. I think this might confuse the issue.



FIGURE 1.—Karyotype of a human female with *cri-du-chat* syndrome. The “marker” chromosome is the *eighth* chromosome from the *left* in the *top row*. It is caused by a deletion in the short arm in one of the chromosomes in the B group. (The break in the *eleventh* chromosome in the *first row* is a technical artifact.)

HISTOCHEMISTRY AND TAXONOMIC ENZYMOLOGY OF CELLS IN *VITRO*

Chairman: Professor John Paul, The University of Glasgow, Glasgow, Scotland

Speakers: Dr. Patricia Farnes, Rhode Island Hospital, Providence, Rhode Island

Dr. Benton B. Westfall, National Cancer Institute, Bethesda, Maryland

Panelists: Dr. Robert S. Chang, Harvard School of Public Health, Boston, Massachusetts

Dr. James L. German III, Cornell Medical Center, New York, New York

Dr. Paul: I must confess to a bit of a disadvantage because I am not sure the title means histochemistry and taxonomic enzymology of cells *in vitro*, but imagine it probably has something to do with differentiation since everything else has. Probably "taxonomic" concerns the classification of cells.

In the old days, people described cell lines in terms of the morphology and tissues of origin. Then, you may remember, people became a bit more scientific about this—karyotypes were introduced, immunology was introduced. Many, to their horror, found that instead of cultivating human tissues, they were culturing new line tissues in the laboratory. I think this explains why very few people have mouse contaminants, because I think most of them have been thrown out quietly.

Most of our criteria seem to have their limitations. In the discussion here, we may get some other indications as to how we may proceed in identifying positively the origin of some of our cell lines.

Histochemical Approaches to Cell Characterization *In Vitro*^{1, 2}

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SUMMARY

Histochemical methodology can be fruitfully applied to investigation of *in vitro* populations. Identification of morphologically ambiguous outgrowth components can be achieved if histochemically demonstrable biochemical properties are retained. Differentiated function in cultured cells may be studied and extents of diversity assessed. Pitfalls commonly encountered include ubiquity of certain histochemical properties,

variability of enzyme activity *in vitro* cells, and technical deficits in histochemical methodology. Examples include the use of histochemical "labels" in marrow cell lineage problems, investigation of metabolic adaptations associated with blood mononuclear-cell modulations, and demonstration of enzymatic "*in situ* diversity" in connective tissue explants.—*Nat Cancer Inst Monogr* 26: 199–228, 1967.

I WOULD like to discuss briefly some of our experiences with histochemical techniques as applied to *in vitro* cells. In contrast to many of the studies presented in this Conference, ours are all short-term cultures of adult human tissues and are primarily concerned with some aspects of fresh tissue—*in vitro* cell relationships. Much of the work described here was accomplished in collaboration with Dr. B. E. Barker.

In at least three areas, histochemical techniques might become uniquely useful in characterization of cultured cells and tissues. These are: First, identification of outgrowth components which, in the course of *in vitro* adaptation, may acquire ambiguous morphologic properties (1–6); second, determination of degree of differentiated function and associated metabolic patterns of cells of established histogenetic origin (7–16); and third, definition of extents of diversity in *in vitro* populations (17, 18).

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HISTOCHEMICAL LABELS

Problems of determining identity of propagating cell types in explant outgrowths are familiar to tissue culturists. Histochemical characteristics or "labels" that could be traced from *in vivo* progenitors to *in vitro* progeny would be useful in identifying look-alike cells engaged in morphologic masquerades. Lord Russell once said, speaking in another context, "The habit of affixing easy labels is convenient to those who wish to seem clever without having to think, but it has very little relation to reality." An ideal histochemical label, to be related to the reality in which we are interested, must be *a unique property of some DNA-synthesizing cell type, which is retained (or continues to be synthesized) by that cell type only, in vitro.*

A few years ago, Dr. Barbara Barker and I became interested in the possible advantages of the histochemical approach to sort out the ontogenetic chaos that has been the lot of bone marrow tissue culture practically from its inception.

In 1928, Harrison (19) remarked with respect to hemic cell culture: ". . . uncertainty still exists with regard to even the most fundamental questions . . ." Nevertheless, he added: "It seems clear that this method will have the last word in clarifying the questions at issue." Nearly 40 years later, the questions—many of them—remain at issue. The stem cell is not yet identified and the last word has not been spoken. Nevertheless, we have an idea from some of our studies of what may *not* be the stem cell and certain aspects of this work may be of interest.

The fibroblast-like cell of bone marrow cultures (fig. 1), which would be promptly classified as a fibroblast in mixed connective tissue outgrowths, has been redescribed with monotonous regularity through the past 56 years in nearly every marrow tissue culture system devised (20–28). Although this cell type has been repeatedly regarded as possessing morphogenetic relationships to nearly every hemic cell series, from morphologic impressions, the extent of its potentialities and all of its possible derivations remain unknown. It is, however, the only kind of cell consistently propagated from normal bone marrow.

I would like to comment here on an interesting point raised in Dr. Gartler's paper, "Genetic Markers as Tracers in Cell Culture." Harris, among others, suggested that Detroit marrow lines might represent other cell types than the originally propagated ones. These cell lines are unique in the annals of marrow culture, since they appeared to undergo epithelial-like transformation after a relatively short period *in vitro*. Similar behavior has not been observed by others in human marrow cultures, and the suggestions of Dr. Gartler are in agreement with the previous speculations.

Studies of a number of investigators, including the recent ones of Berg and Rosenthal (25) and Ang and associates (26), appear to have been

predicated on the hope that these fibroblast-like cells bore some relation to hematopoietic elements. Benyesh-Melnick and associates (27) suggested (but never definitively showed) that "blastoid transformation" of such cells from leukemic marrows might occur. The problem is complicated by evidence for long lymphocyte survival *in vitro*, which could serve as a source for the blastic colonies described (28). If the fibroblast-like cells bear the relationship to hemic cells attributed to them by Foot (21), Lajtha (29), or Reisner (30), they must represent near relatives of the stem cell *in vitro*, and all we must do to cultivate the hemic cells themselves is to find some way to redirect these fecund rebels into differentiating channels.

Certain facts suggest that the fibroblast-like cells might be derived from stroma, as originally proposed by Carrel and Burrows (20), Maximov (31), and more recently by Berman and others (24). The cells arise from margins of explants. Plasma cells are frequently seen in association with them in early outgrowths, and the close relationship of plasma cells to vasculature, described by Ranvier, has been repeatedly confirmed in bone marrow. Likely precursor cells of the fibroblast-like elements might include fat cells, capillary endothelium, "macrophages" or "reticular" cells, pericytes, and a variety of fibroblasts—common, adventitial, or periosteal. Bone particles are rare in the aspirated marrow we have used in our studies, and blood vessels larger than capillaries are only occasionally encountered (32).

The distribution of a number of histochemically demonstrable enzymes in fresh marrow and cultured outgrowths cells is shown in table 1. Enzymes associated with the pentose pathway, Krebs cycle, and glycolytic pathway, as well as adenosinetriphosphatase and L-leucynaphthylamide aminopeptidase, were demonstrated in all suspected precursor cell types, and were therefore valueless as labels of particular cell types. Others, exemplified by monoamine oxidase and glucose-6-phosphatase (not listed here), were inappropriate because they were not demonstrated in any cell types.

"Absence" of a histochemical characteristic, as used here, refers only to a lack of a demonstrable level of activity of that property under a standard histochemical technique. Although levels below the threshold of sensitivity of any given method may exist for some of the substances in question, it is convenient and well precedented to speak of absence of detectable enzyme activity in terms of any visible product under these controlled circumstances.

One esterase, demonstrated by naphthol-AS acetate, was ubiquitous and of no value as a label, but α -naphthyl acetate esterase was restricted to certain cell types, in the explant and in the outgrowth. These differences in localization of enzyme for esterase and β -glucuronidase might be attributable to differences in sensitivity of techniques or to differences in specificity of the methods. In enzyme families composed of a number of

TABLE 1.—Comparative histochemical properties of fibroblast-like cells and possible progenitors from marrow stroma

Parameter	Capillary*	Histiocytes	Fat cells	Mono- cytes	Peri- cytes	F-LC†
Alkaline, phosphatase.	+	0	0	0	0	+
Acid phosphatase.....	0	+	—	+	0	0
Esterase (α NA).....	0	+	—	+	0-trace	0
Esterase (NASA).....	+	+	—	+	+	+
β -Glucuronidase (NASBI).....	0	+	—	+	0‡	0
β -Glucuronidase (8-HQ)	+	+	—	+	+	+
Adenosinetriphos- phatase.....	+	+	—	+	+	+
Aminopeptidase (β - LN).....	+	+	—	+	+	+
Dehydrogenases: SD, LD, β -OHBD, G6PD, glutamic D.....	+	+	—	+	+	+

* Includes endothelial cells and so-called "reticular" elements in vascular network.

† Histochemical properties during emergence from explant and first 5 days of culture.

‡ Closely associated plasma cells reactive for enzyme.

isozymes, which include many of the properties described here, differences in activity of various cell types may reflect variations in isozyme distribution.

α -Naphthyl acetate esterase, acid phosphatase, and naphthol-AS-BI glucuronide β -glucuronidase were present in marrow histiocytes and in cultured "macrophages," but absent in endothelium and in fibroblast-like cells. Alkaline phosphatase distribution was consistently demonstrated in capillary endothelium (fig. 2) and in terminal cells contiguous with the vascular network, which may be at the level of the capillary and sinusoidal junctions. This enzyme was also consistently shown in all of the fibroblast-like cells in the outgrowth, as early as cell attachment could be seen (fig. 3). Diligent searching revealed no activity by any technique employed in "macrophages," lipocytes [suggested by Miyoshi and others (33) as parents of marrow fibroblast-like cells (fig. 4)], or pericytes, for which no distinctive label has been found and whose *in vitro* fate is still obscure.

In the search for some unique property of the pericyte, we were encouraged by Hayashi's results with rat tissue (34), in which pericytes of brain capillaries contained strong β -glucuronidase activity. Trials of this technique in fresh human bone marrow showed that endothelial cells and pericytes showed no activity. Instead, densely staining cells in perivascular location, but external to the perivascular sheath, proved to be plasma cells (fig. 5).

Activity of alkaline phosphatase was not acquired by other proliferating or vegetative cell types in the outgrowth. Monocytes, which can spindle out to resemble fibroblast-like cells in blood and marrow cultures (fig. 6), were never reactive. Fat cells rarely entered the outgrowth at all under the conditions of our cultures, but remained nonreactive in the explant. "Macrophages" were never reactive for the enzyme, even with prolonged incubation and the use of most sensitive techniques (35).

The fibroblast-like cells in early outgrowths of marrow appear to be derived from alkaline phosphatase-reactive vascular elements of marrow stroma, which probably correspond to the common endothelium referred to by Maximow (36). There is no reason from the information at hand to suppose that these cells have any relationship to hematopoietic stem cells, which are not known to be characterized by alkaline phosphatase activity. The possibility that an alkaline-phosphatase-reactive precursor serves as an ultimate source for a hemocytoblast cannot be excluded, but there is no evidence to support such a notion at present. In any event, it is obvious that the fibroblast-like cells in these early cultures do not arise from a variety of early hemic cell types, as has been claimed by some investigators.

The observation that histochemical properties of early outgrowth cells may be influenced by variations in the milieu will come as no surprise to any investigator. Although histochemically demonstrable alkaline phosphatase activity of mature myelocytic cells *in vitro* appears to be medium dependent (37), alkaline phosphatase activity of emigrating marrow fibroblast-like cells could be demonstrated in every medium tested, including a variety of chemically defined media, supplemented with several homologous or heterologous sera. Histochemical and morphologic changes could, however, be invoked in these cells by addition of glucocorticoids (38).

Compared to fibroblast-like cells in control cultures, those in prednisolone-treated cultures show increased cell size, increased nuclear size, and increased nucleolar size and number (fig. 7). This is accompanied by marked cytoplasmic basophilia. Toluidine blue staining with ribonuclease digestion of split coverslips indicates that the basophilia is due to increased RNA in the cytoplasm of the steroid-treated cells.

Certain other properties of the steroid-treated cells are susceptible to histochemical demonstration. In prednisolone-treated cultures, persistent association of fibroblast-like cells in capillary arrangement can sometimes be noted during the first week of culture (fig. 8). This phenomenon might be related to enhancement of capillary stability produced by glucocorticoids *in vivo*, but in any event incidentally results in microenvironments of high population density. Alkaline phosphatase activity, which appears to decrease in marrow fibroblast-like cells during the second week of cultivation in our cultures, appears more intense in the steroid-treated cells.

In aging cultures without hormone, acid phosphatase and α -naphthyl acetate esterase appear in the fibroblast-like cells after about a week, but the appearance of these enzymes at histochemically demonstrable levels is retarded by addition of steroids, an effect which could be related to lysosomal stabilization (39).

Glycogen, known to be induced by glucocorticoids in certain mammalian cells (40), is also increased in the steroid-treated fibroblast-like cells (fig. 9). Endothelial cells are one of the many *in vitro* cell types capable of glycogen synthesis, and this underscores the nonspecificity of glycogen-synthesizing capacity as a marker for any particular cell type *in vitro*, a point noted by others.

Studies of some other histochemically demonstrable properties of these cells showed no significant effects of the hormone. The activities of succinic, malic, lactic, and glucose-6-phosphate dehydrogenases showed no consistent changes between control and steroid-treated cells. This does not prove that changes in the metabolic pathways sampled by histochemical assessment of these enzymes did not occur; significant changes of an order of magnitude undetectable by these histochemical reactions might have been present. In addition, it is important to note that significant variability in cell-to-cell reaction occurred consistently in most cultures studied.

The effects of prednisolone that were observed might be the result of specific independent mechanisms, such as induction of synthesis of particular enzymes (40-42). Or, they might be symptoms of more fundamental mechanisms by which a variety of specialized cell functions—of which these are a few—are augmented.

All that spindles is not a fibroblast, and histochemical approaches may be valuable in sorting out mixed spindled populations *in vitro*. For example, in acute monocytic leukemia, a mixed population may arise from the explanted marrow. The normal stromal elements are joined by leukemic monoblasts which exhibit a high degree of polymorphism: They may appear as "macrophages," multinucleate giant cells, or spindled cells which show superficial resemblances to fibroblast-like cells.

Many years ago, Strangeways (43) provided detailed morphologic observations to refute Maximov's interpretation of "fibroblastic" appearance of cultured monocytic cells. He concluded that morphologic resemblance of monocytic cells to stromal cells *in vitro* was a superficial one. In general, this is true of normal human-blood monocytes and stromal elements in our culture system as well. Nevertheless, some leukemic monoblastic cells bear extraordinary resemblances to connective tissue elements in human blood and marrow cultures and, in such instances, histochemical differences can prove valuable in cell identification. In one case we studied leukemic monoblasts that morphologically resembled stromal elements but which differed from them by lack of alkaline phosphatase activity and presence of strong acid phosphatase and esterase activity (3).

Histochemical labels must be synthesized or retained by the parent cell *in vitro* but must not be acquired by other types of cells in the outgrowth. Mechanisms by which such appearance of label in a foreign cell type might occur must be taken into account; such properties might spread about by active transport from one cell type to another, phagocytosis, pinocytosis, emperipolesis with subsequent death of the labeled cell, or induction of enzyme synthesis in a formerly nonreactive cell. An interesting example of the sort of problem which may arise is exemplified by some of the studies of melanin-producing cells *in vitro*. In such cultures, melanin granules have been described as being phagocytized by macrophages and other cell types (44) and undergoing transportation from melanocytes to Malpighian cells (45). The recent work of Nakai and Shubik (46) has shown that not only melanin, but tyrosinase, which is associated with the melanosome in the synthesizing cell, enters macrophages.

One of the most serious objections to the use of such approaches as histochemical labeling *in vitro* is, of course, that rapid loss of *in vivo* properties often occurs. The well-known proclivities of some liver and kidney parenchymal cells to lose activity of certain specific enzyme systems after brief explantation are examples of such considerations (47-49). Histochemical studies of fresh tissue outgrowths might prove ideal in some instances for exploration of factors influencing retention and loss of certain differentiated properties *in vitro*.

MODULATION IN VITRO

Histochemical techniques are particularly well suited to convenient demonstration of parameters of differentiation, maturation, and modulation in small populations in culture. The relationship of morphologic and biochemical manifestations of these processes has been studied in a number of tissues, including bone (8), muscle (9-11), blood cells (12-16), and other cell types (40, 41, 50).

Theoretically, histochemical studies might be of unique value when a single cell type is capable of a variety of modulative pathways that may be associated with different metabolic adaptations. Blood lymphocytes, for example, have been nominated for participation in macrophage formation (51-54), fibroblast formation (55), conversion to hemic cell types, and transition to globulin-producing immunocytes (56). Whether the lymphocyte will finally live up to all of these expectations is an intriguing question far beyond the scope of the discussion at hand. However, comparative studies of histochemical attributes during any of these transitions (or circumstances under which they are believed to occur) might yield important information about biochemical activities associated with these functional states.

Detailed histochemical studies have already been made, for instance, on blood lymphocytes undergoing blastogenesis in response to PHA, the phytohemagglutinin from *Phaseolus vulgaris*. Under these conditions, small blood lymphocytes undergo a series of morphologic changes *in vitro*, notably hypertrophy of nucleus and cytoplasm, marked cytoplasmic basophilia, nucleolar prominence, and active mitosis. This process, inappropriately termed "transformation," will be referred to as blastogenesis. Quaglino and others showed that the blastogenesis in response to PHA was associated with several alterations in metabolism, including increase in glycogen, and active glycolysis (13). Activity of other key dehydrogenases also increased during the process. Although these workers failed to demonstrate fat in cells undergoing blastogenesis, it is now established that lipid accumulation is extensive during the preblastic phase. It has also been shown that this process is accompanied by synthesis of large amounts of RNA.

The Hirschhorns and their associates showed that acid phosphatase and β -glucuronidase were increased prior to mitosis of these cells (14, 15) and subsequently postulated that activation of lysosomal enzymes might play a role in initiation of mitosis (57). More recently, Tanaka and Liddy clarified the localization of acid phosphatase activity by showing that cytoplasmic vacuoles of the lymphocytes were lipid and that lysosomal enzymes were localized outside of the vacuoles (figs. 10 and 11) (16).

In 1964, we discovered a second phytomitogen in *Phytolacca americana* (58). Since then we have performed comparative histochemical studies of lymphocytes undergoing blastogenesis in response to PHA or PWM (pokeweed mitogen). Such studies are of particular interest since Douglas and associates (59) suggested that ultrastructural differences may occur between cells responding to the two mitogens, observations which have not to this date been confirmed. Details of the histochemical studies will be reported elsewhere, but it may be of interest to note here certain metabolic effects which are common to blastogenesis in either instance, and which have been largely elucidated through histochemical methodology.

Glycogen synthesis and degradation are increased during the premitotic period. Similarly, fat accumulation in the cytoplasm of the preblastic cells is increased to a marked degree. This is accompanied by an increase in a number of dehydrogenases, among them β -hydroxybutyric dehydrogenase (fig. 12). These observations suggest that lipid metabolism may play an important role in the blastic process.

Comparison of these histochemical properties with those of peripheral blood mononuclear cells undergoing macrophage formation indicates that there is nothing specific about some of the changes (table 2). In the right-hand column, parameters are listed which apply to human blood monocytes

undergoing macrophagogenesis. Lymphocytes in such cultures, which may also participate in macrophage formation (60-62), show moderate increase in acid phosphatase (fig. 13) and β -glucuronidase. These changes can hardly be related to initiation of mitosis, since these cells do not divide. As Miss Ackley has shown in our laboratory, there is also an increase in succinic, lactic, and glucose-6-phosphate dehydrogenase activity in these developing macrophages (figs. 14 and 15).

TABLE 2.—Comparative histochemistry of two *in vitro* processes of blood mononuclear cells

Parameter	Blastogenesis	Macrophago- genesis
Alkaline phosphatase.....	0	0
Acid phosphatase.....	++	++++
β -Glucuronidase.....	++	++++
Glycogen.....	Increase	Increase
Succinic dehydrogenase.....	Increase	Increase
Lactic dehydrogenase.....	Increase	Increase
Glucose-6-phosphate dehydrogenase.....	Increase	Increase
DNA synthesis.....	+	0
Lipid.....	Increase	Increase

Bearing in mind that there are enormous differences between chickens and people, it is interesting to examine some of this data in terms of Dr. Sutton's ultrastructural studies of giant cell formation *in vitro*. Human blood cells form multinucleate cells in culture also, and these exhibit the histochemical profile shown in the table, with the exception of glycogen, which we have not studied. The prominence of lysosomal and mitochondrial enzyme activities leads us to suspect that ultrastructural studies of human giant cells might show some resemblances to those of the chicken. I hope that someone will undertake such studies, since knowledge of the interactions and fine structure of lymphocytes and monocytes in human cultures would be important.

It seems clear that the relationships of biochemical attributes of cells undergoing blastogenesis or macrophage formation to critical functional alterations in these processes remain to be defined. One approach that might be interesting is exploitation of unique metabolic blocks occurring in leukocytes in certain congenital nonspherocytic anemias and in other inborn metabolic errors. Specifically, studies of blastogenic and macrophagogenic capacities and the characteristics of these modulations in leukocytes from patients with deficits of triosephosphate isomerase (63), phosphorylase (64), amylo-1,6-glucosidase (65), and glucose-6-phosphate dehydrogenase (66) might provide information about critical control mechanisms for either process.

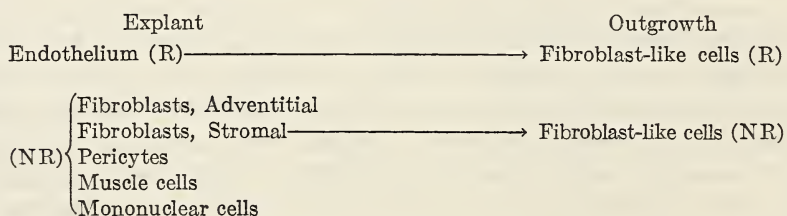
"IN SITU" DIVERSITY

At the Syverton Memorial Symposium in 1961, Kahn and associates presented a discussion of some histochemical studies in L929 cells, in which they found wide diversity in degree of phospholipid content and several dehydrogenases (17). From these and other studies, both biochemical and histochemical, it seems that such diversity, with respect to many parameters, is usual in many types of *in vitro* populations.

A great amount of attention has been given to factors that might contribute to such diversity, of which the discussions of Kahn, Eagle (67), Paul (68), and others are representative. I would like to discuss briefly a point which is rarely mentioned very critically; that is, the importance of sorting out "fibroblasts" and the like in mixed explant populations.

In situ diversity, which precedes all of the other sorts of diversity, begins in the explant and depends upon *in vivo* genetic and epigenetic factors. The extent of this diversity may in turn influence the ultimate extent of diversity which occurs in the *in vitro* milieu. For instance, it is not known whether progeny from an adventitial fibroblast, nonreactive for alkaline phosphatase *in vivo*, may be expected to carry on enzyme synthesis with the same frequency, intensity, or duration after 20 passages *in vitro* as a reactive endothelial cell.

Two years ago we reported some studies of skin and hernial sac cultures which demonstrated that a consistent population split could be defined in initial outgrowths of mixed connective tissue, with respect to histochemically demonstrable alkaline phosphatase activity (69). We studied explants and outgrowths with a method combining tritiated thymidine autoradiography and the histochemical alkaline phosphatase reaction of Gomori (70). As shown below, the listed cell types synthesized DNA, demonstrated by incorporation of nuclear tritiated thymidine, in the explant: capillary endothelium (reactive for alkaline phosphatase), and adventitial fibroblasts, pericytes, smooth muscle, and occasional blood mononuclear cells (all nonreactive for the enzyme). In the outgrowth, alkaline-phosphatase-reactive and nonreactive cells appeared side-by-side (figs. 16 through 19).



Connective tissue populations derived from explants of skin, synovial membrane, hernial sac, and breast connective tissue all behaved similarly. It appears reasonable to conclude that with respect to alkaline phosphatase activity at least two sorts of morphologically similar cells differing in

activity of this enzyme arise routinely from explants of connective tissue. The progenitor cells of these two types in the explant also appear to differ from one another in this property. Reactive cells from the same tissue specimen constituted up to 95% of the population in some cultures and as low as 4% in others. It is evident that the composition of outgrowth, in terms of cells demonstrating enzyme synthesis under these culture conditions, is unpredictable.

If histochemical methods are to contribute useful information about metabolic properties of cultured cells, some means of accurate quantitation will inevitably be required. A number of techniques have been devised to relate histochemical and biochemical data on a quantitative basis. These include the Coujard dilution technique (70), autoradiographic labels to quantitate precipitates (71), extraction and protometric measurements of products, and interference microscopy (72). Accurate quantitation of histochemically demonstrable properties in enzymes depends on conditions in which substrate concentration is high enough to insure zero order kinetics. Moog has shown that activity of phosphatase could be expressed as a function of time of first appearance of product under such circumstances.

Benditt and Arase (73) showed that the Lineweaver Burke expression of the Michaelis Menten equation describing kinetic behavior of an esterase in mast cells could be applied to a histochemical reaction in tissue sections. These results indicate the feasibility of mathematical expression of histochemical data. In addition, a general quantitative agreement between average levels of activity assessed histochemically and biochemical analyses in cultured cells has been noted for several enzymes (15, 74). In some studies, defects in sensitivity of certain histochemical methods have been clearly shown as well (75).

Nevertheless, attempts to quantitate enzyme activity or other histochemical properties in cultured cells may provide misleading data unless cell-to-cell variation is taken into account. Validity of quantitation techniques, except those dealing with individual cells (76, 77), depends upon homogeneity of the population. It might be possible to ignore flask-to-flask variation expressed in terms of hydrolysis of μ moles substrate per unit protein per unit time, but if we know that the cells really look like this (fig. 20) we may have reservations about interpretation of results. Even attempts to quantitate, by measure, the time of first visible product would yield only the scope of activities present.

One great advantage of the histochemical approach is that it permits localization of products to subcellular organelles. Thus, activity of an enzyme may be assessed, and the number, size, and structural attributes of the associated organelle may be visualized. The combination of histochemistry with ultrastructural techniques has been particularly effective in this regard. Such observations, especially when made in critically designed

experiments, are apt to be of broad biologic significance in cultured cells, because they represent a unique way to relate structural and physiologic properties of individual cells. The accuracy of localization is a function of the total quality of histochemical technique employed. Erroneous localizations of acid phosphatase to fat vacuoles, alkaline phosphatase to lysosomes, and soluble fraction dehydrogenases to mitochondria have been reported. On the other hand, localization of a number of histochemical properties on a cytologic level has been achieved (9-11, 16, 78-80).

One of the chief virtues of histochemical perspective is that by use of its methods, we are able to learn about specific properties of specific cells, rather than confine our thinking to average properties of average populations of cells. The dimensions which could be realized with this approach depend upon appropriate and thoughtful combination of histochemical methods with other techniques and, of course, with fruitful ideas.

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PLATE 38

FIGURE 1.—Fibroblast-like cells from a 10-day marrow culture, showing typical spindled orientation. Mitotic figures are present. Wright-Giemsa. $\times 100$

FIGURE 2.—Alkaline phosphatase reaction of normal capillary network of marrow. Individual reactive "reticular" cells, most of which appear to be associated with the termination of the capillary network, are present. (Gomori azo technique, Diazo Blue B), Toluidine Blue. $\times 100$

FIGURE 3.—Alkaline phosphatase reaction of 3-day-old fibroblast-like cell in normal marrow culture, showing strong cytoplasmic reaction. (Gomori azo technique, Fast Blue RR), Nuclear Fast Red. $\times 500$

FIGURE 4.—Alkaline phosphatase activity in marrow explant 4 days *in vitro*, cultivated in presence of prednisolone. Capillary is intact and reactive for enzyme. A large highly vacuolated cell is present (*arrow*) which resembles fat cells suggested as fibroblast-like cell precursors. This fat cell shows no alkaline phosphatase activity. (Gomori azo technique, Diazo Blue B), Nuclear Fast Red. $\times 2,400$

FIGURE 5.—Demonstration of β -glucuronidase activity in human bone marrow binucleate plasma cell, Hayashi technique. $\times 1,500$

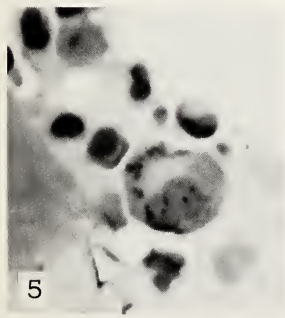
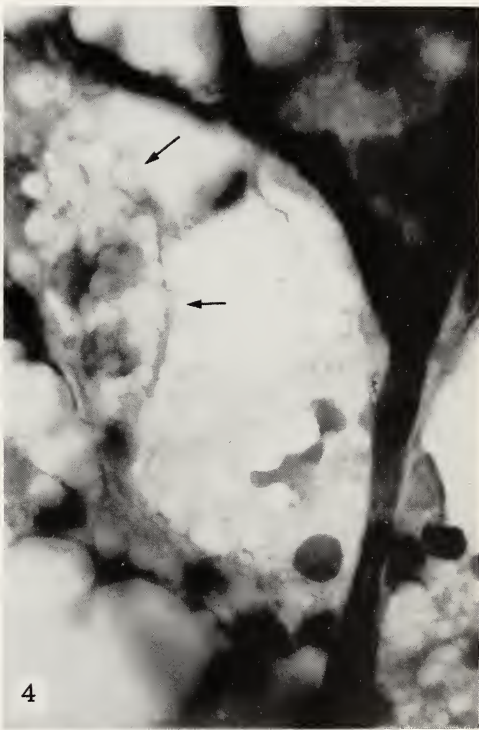
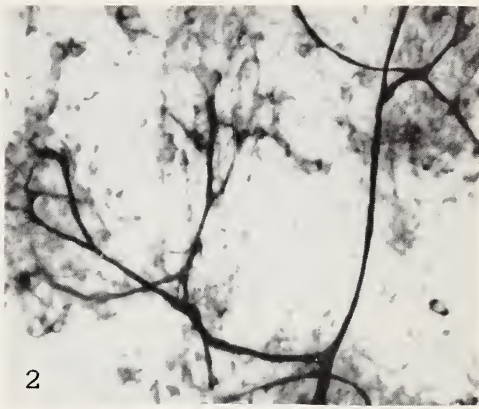
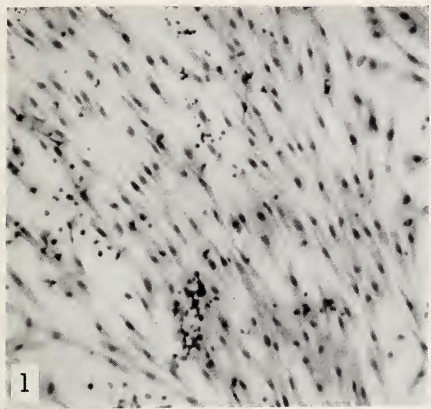


PLATE 39

FIGURE 6.—Alkaline phosphatase reaction in cultured peripheral blood cells in a 5-day culture, showing no activity of this enzyme. The mononuclear cells vary in shape, some superficially resembling fibroblasts, and others rounded. (Gomori azo technique, Fast Blue RR), Nuclear Fast Red. $\times 1,000$

FIGURE 7.—(a) Four-day control culture of marrow, showing small number of fibroblast-like cells and hemic cells. (b) Same marrow with 5 $\mu\text{g}/\text{ml}$ prednisolone added to medium. There is increased cellularity; fibroblast-like cells are larger, with large nuclei and prominent nucleoli. Wright-Giemsa. $\times 100$

FIGURE 8.—Aggregate of fibroblast-like cells from a prednisolone-treated culture, showing arrangement similar to capillary architecture. There is strong alkaline phosphatase activity in all cells. (Gomori azo technique, Fast Blue RR), Nuclear Fast Red. $\times 500$

FIGURE 9.—(a) Periodic acid-Schiff (PAS) reaction in control fibroblast-like cells (4 days) showing particulate positive material. (b) Strong PAS reaction (diastase labile) in same marrow cells cultured with prednisolone. (PAS with no diastase, hematoxylin). $\times 500$

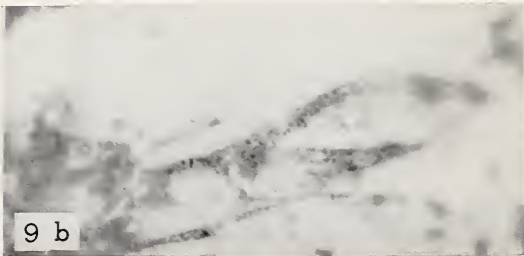
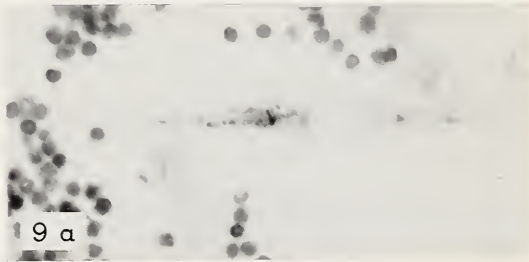
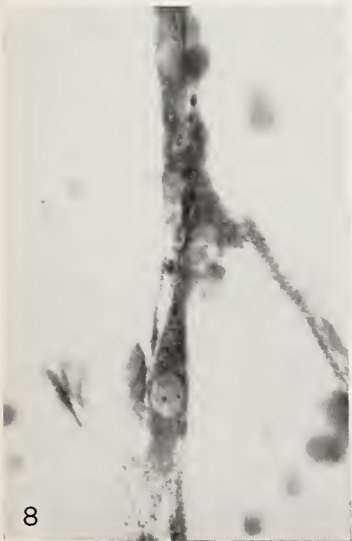
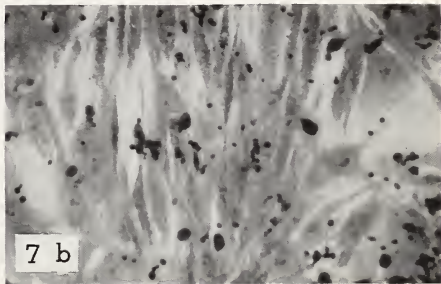


PLATE 40

FIGURE 10.—Acid phosphatase reaction in 48-hour cultured human blood cells, with pokeweed mitogen. Fine grains of activity are distinct from vacuoles. (Gomori acid phosphatase), Nuclear Fast Red. $\times 2,000$

FIGURE 11.— β -Glucuronidase reaction in 48-hour culture with mitogen. Lymphocyte shows product distinct from cytoplasmic vacuoles. (Naphthol AS-BI glucuronide), Methyl Green. $\times 1,000$

FIGURE 12.— β -Hydroxybutyric dehydrogenase reaction in 72-hour cultured cells, with mitogen. There is strong reaction in mitotic cell and other mononuclear cells. (Barka and Anderson), Nuclear Fast Red. $\times 500$

FIGURE 13.—Acid phosphatase activity in 5-day cultured macrophage from peripheral blood without mitogen. There is strong particulate cytoplasmic activity. (Naphthol AS-TR phosphate), Methyl Green. $\times 2,000$

FIGURE 14.—Lactic dehydrogenase activity in 7-day cultured peripheral blood cells, without mitogen. Macrophage and giant cell formation is associated with increased activity of this enzyme. (Barka and Anderson), Nuclear Fast Red. $\times 750$

FIGURE 15.—Succinic dehydrogenase activity in macrophages, some of which show superficial morphologic resemblance to fibroblasts, and giant cells in 7-day peripheral blood culture, without mitogen. Linear distribution is probably associated with mitochondrial localization. (Barka and Anderson), Nuclear Fast Red. $\times 150$

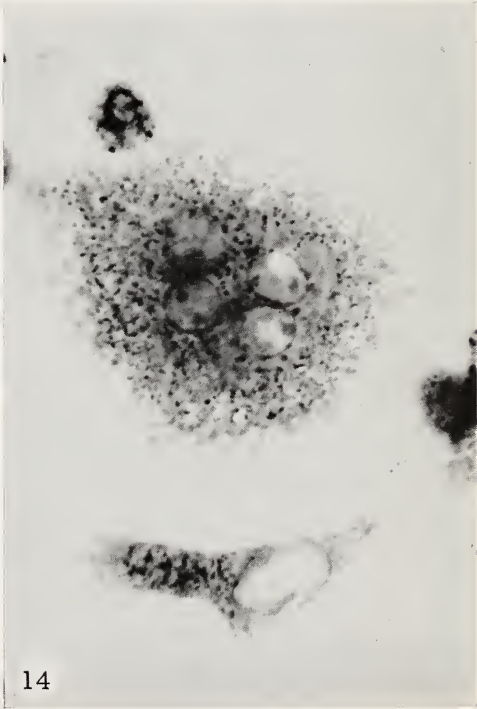
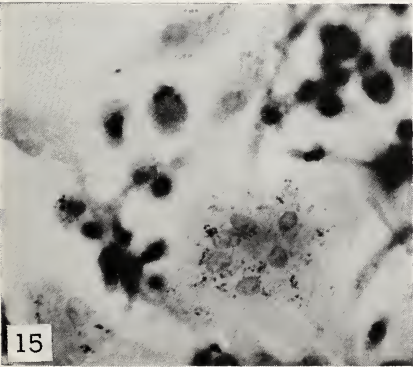
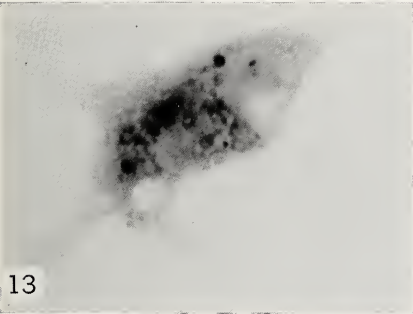
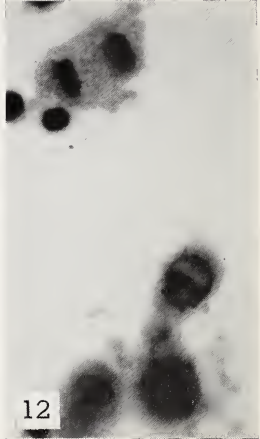
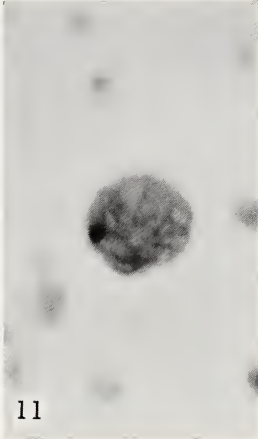
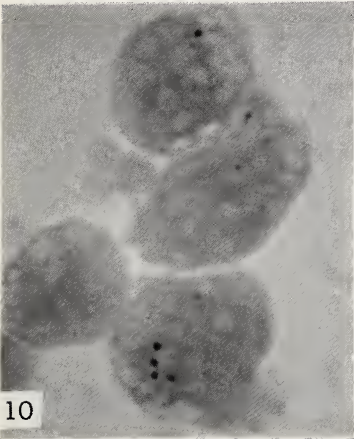


PLATE 41

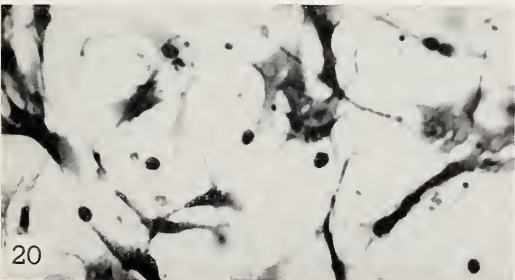
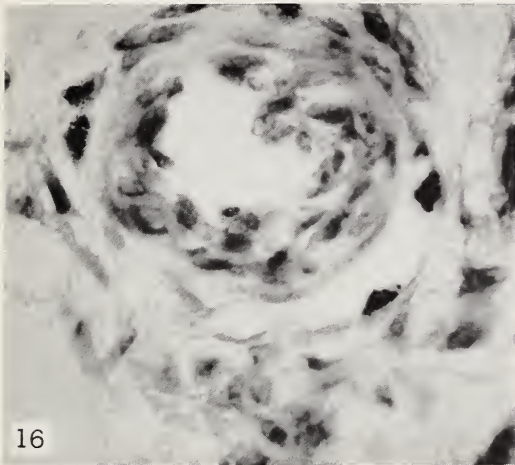
FIGURE 16.—Combined autoradiograph-alkaline phosphatase reaction in 3-day hernial sac explant, showing a nonreactive artery with thymidine labeling of adventitial fibroblasts, not reactive for alkaline phosphatase. Toluidine Blue. $\times 600$

FIGURE 17.—Similar area of another explant, showing alkaline phosphatase reactive intact capillary, with a perivascular cell, nonreactive for enzyme, showing nuclear incorporation of thymidine. Toluidine Blue. $\times 1,000$

FIGURE 18.—Four-day hernial sac explant, combination tritiated thymidine autoradiograph-alkaline phosphatase reaction. Reactive capillary cells are dispersing and are synthesizing DNA as indicated by nuclear thymidine incorporation. Toluidine Blue. $\times 1,000$

FIGURE 19.—Outgrowth from 3-day hernial sac culture (combination autoradiograph-alkaline phosphatase reaction). Strongly reactive cells and completely nonreactive cells are present side-by-side in the outgrowth. Both cell types incorporate tritiated thymidine. Morphologically, the two cell types appear identical. Nuclear Fast Red. $\times 1,500$

FIGURE 20.—Combined autoradiograph-alkaline phosphatase reaction on skin outgrowth zone (5 days). Variation in activity from nonreactive to strongly reactive is evident in morphologically similar cells. Nuclear Fast Red. $\times 150$



DISCUSSION

Dr. Paul: Thank you, Dr. Farnes, for this very detailed review of the role of histochemistry in identifying the origin of some of our cells.

Dr. Pearse: As you have just heard and seen, histochemistry and cytochemistry together constitute the science of localization of substances and functions in tissues and cells. The localization is the important point. And, in fact, the true words should be "histotopochemistry and cytotochemistry." The added syllable is derived, not from the Italian for "mouse," but from the Greek, "topos," meaning place or location.

The two main branches are 1) functional (histoenzymology) and 2) analytical. The whole science is full of deficiencies. Dr. Farnes has mentioned some of them. The main one is usually supposed to be quantitation or the lack of it. Some people even define cytochemistry as the quantitative science, leaving histochemistry as a kind of dabbling with the microscope. This view is absolutely anathema to me. Histochemistry, and cytochemistry which is included in it, are one and the same, by definition.

Quantitation will come. It is a matter of difficulty of instrumentation, and the tediousness and difficulty of some of the more beautiful techniques such as those depending on microdissection and microassay.

The next deficiency, which is at one time a deficiency and not a deficiency, is the threshold (sensitivity, or lack of sensitivity) of the reactions, particularly the enzyme reactions. This enables us to get answers about these reactions in black and white, whereas biochemical answers would come out as various shades of gray. It is often easier to interpret black and white.

Technical limitations are particularly evident in the enzyme field. A large number of enzymes have already been described, and there are perhaps 350 or so reasonable ones. There are over 700 enzymes, but some of them, of course, are only present in organisms which convert lead to gold and things of that sort.

If there are 350 sensible ones, we can now demonstrate about 65 reasonably well. So here is another very grave deficiency. It means that we cannot choose the system we would prefer in order to fit any given cell. We have to keep within the frame of reference of those 65 techniques. Dr. Farnes said we had to keep well within the 65 or we would be here all night.

The other deficiency she covered is the one shared by cytologists. I remember saying some years ago at a meeting of cytologists in Detroit that they had to take full responsibility for failing with my beautiful techniques by using them on air-dried smears, where there is an intact cell and an intact cell membrane, as there is presumably in tissue culture also. This intact membrane only adds to one's troubles because histochemists and cytochemists usually use sections in which at least a proportion of the cell membranes has gone, though of course the organelle membranes are still probably intact. The importance of these organelle membranes cannot be overestimated because, with the methods of enzyme cytochemistry used by Dr. Farnes, the true rate-limiting factor is often not the level of enzyme or co-factor, but the rate of penetration of these membranes by the reagents used.

The point I wish to make is that advances in technology have made it possible to demonstrate histochemically five or six of the acid hydrolases which are contained in lysosomes. The object I am using is the pituitary gland of the newt, and it is quite clear that there are different appearances, and a different view of the lysosome population, with the different enzyme systems. The acid phosphatase technique has been for some time the classical marker for lysosomes. Probably it should no longer be used as such. The four enzymes that I am comparing are a) acid phosphatase, b) β -glucuronidase, c) β -glucosaminidase, and d) aryl sulphatase. It should be evident that an entirely different population of lysosomes, if you accept that they are

lysosomes, is picked out by the four methods I have chosen. Two of the techniques, that for acid phosphatase and the one for aryl sulphatase, can be adapted for use at the electron microscope level. At this time I have applied only the acid phosphatase technique, and this shows, in the globoid cells of the newt pituitary gland, that acid phosphatase presents only in the larger particles.

Another application of these techniques has been to carry out an examination of various populations of lymphocytes, particularly in the thymus and in lymph nodes and spleen. The lymphocyte classically has a single lysosomal particle, or nothing at all, when the acid phosphatase technique is used. Again, with β -glucosaminidase, the thymus lymphocytes normally show a single lysosomal particle. Many more seem to be revealed, however, than by the classical acid phosphatase technique. In the lymphocytes of the spleen, the second technique shows as many as two or three lysosomes per lymphocyte. This indicates a difference from those of the thymus.

Finally, in histochemistry one should never use one method where two exist, or two where three exist, or three where four, and so on, *ad infinitum*. It is always safer to use a barrage of methods. To illustrate the point that one can show lysosomes in lymphocytes and elsewhere by other techniques, some of the slides showed lymphocytes that have taken up injected peroxidase. This is the technique of Werner Straus. The ingested peroxidase is demonstrated by the benzidine reaction, and this can also be adapted for use with the electron microscope.

One can also demonstrate a population of lysosomes without using any enzyme technique or, for that matter, any staining technique. Lysosomes, when fixed by the application of formaldehyde vapor to freeze-dried material, are autofluorescent. They fluoresce in different colors in different tissues and I think also in different states in the same tissues or cells. These results require instrumental analysis.

Now I would like to ask Dr. Farnes a few questions:

1) Is it true that enzyme activity, for example, alkaline phosphatase as mentioned in your abstract, is not acquired during culture by cells that did not have it in the first place?

2) I was impressed by the fact that you could not find monoamine oxidase in any cells. Do you think it might be possible to develop enzyme activity by giving them a substrate to work on? For instance, if you added, say, tryptamine to the medium, I think that certain cells might be induced to deal with it by developing monoamine oxidase, and thus perhaps they would show their nature.

I am suggesting this broadly as a possible way of sorting out cells by making them do something they normally do not do because, in the beautiful media you put them in, they do not need to do it.

3) This is really part of the first question. Is it not more profitable sometimes to demonstrate an active mechanical property of the cell by making it phagocytose something? I am thinking of work like that of Holmes and Watson, who have shown that thorotrast provides a very nice electron-dense marker for hamster kidney cells in tissue culture. A tremendous number of cells do apparently phagocytose things even though they do not normally do it in their ordinary environment.

Casley Smith, for instance, has shown that HeLa will take up fluorescent γ -globulin. They would no doubt take up peroxidase too.

4) Can you substantiate your identification of the β -glucuronidase-positive cells as plasma cells? I am very alarmed about this. I have never succeeded in making a plasma cell do anything like that. In fact, it is a very boring cell from the point of view of the enzyme histochemist. I would be glad to know whether you are absolutely certain of the identification. Personally, I would conclude that a cell containing β -glucuronidase was not a plasma cell at all.

5) In my fifth question there are four points, but only two that I really want to make, so we'll skip the other two. (a) Have you considered using phenazine meto-

sulphate (PMS) to increase the chances of detection of various dehydrogenases that are below the threshold level unless you use additional acceptors? As a corollary to this you might consider it in the case of soluble dehydrogenases which some people say they can localize, whereas others deny it. (b) Finally, I do not really think much of your isozyme hypothesis to explain histochemical differences. There are so many other reasons for these differences. Histochemical techniques for instance will show in many tissues two different alkaline phosphatase when very thorough biochemical studies show that there is only one enzyme. So histochemistry should not detect two, even if it does.

Histochemistry may also be defined as the production of visible artifacts in cells and tissues. We employ very unnatural substrates, and electron acceptors, etc. Tetrazolium salts for instance, without which we cannot demonstrate dehydrogenases, effectively block all oxidative phosphorylation. One cannot comment on that function one way or the other when a system containing tetrazolium salts is used.

Other reagents affect the state of membranes such as the lysosomal membrane. They change its permeability, and they certainly affect the recorded level of acid phosphatase; that is, we get more product. And if we had an instrument that could estimate the amount of product in the cell, we would say there is more acid phosphatase, but clearly we don't produce any more acid phosphatase by our procedures. We can only hope that after we have carefully standardized our procedure the changes we record may reflect, unaltered, the *in vivo* status.

Vivianne Maggi, for instance, suggests that the raised acid phosphatase in mitosis reflects increased permeability of the lysosomal membrane rather than increase in enzyme. I think she may well be right about it, too.

Dr. Farnes: As you gathered from my presentation, the problems I am concerned with are primarily those of cell relationships. Therefore, I did not go further than necessary to make the points in which I was interested. As to the first question, we were very cautious. I tried to emphasize that we studied these enzymes both in the explant and in the outgrowth at extremely short intervals and that we simply were never able to demonstrate this in any other type of cell. Being very suspicious, we cultured normal bloods at the same time from the same people and found that none of the hemic cell types present in peripheral blood, including lymphocytes and monocytes, cultured under identical conditions, developed this activity, although morphologically they could resemble the cells in question.

I have made no effort to induce enzyme activity or tease the cells into responding in the manner suggested here. It might be quite a rewarding approach under certain circumstances, but not in the ones at hand. We demonstrated a battery of differences, and it seemed unnecessary to resort to attempts to manipulate enzyme-synthesizing activities. Interpretations are really difficult enough as it is.

In my cultures, cells of many sorts phagocytized. This is one reason that I would not rely on such a characteristic for cell type identification, because phagocytosis also occurs in fibroblast-like elements as well as in the typical macrophages or histiocytes, as we might call them. This is not a specific property in this culture system, and illustrates precisely the need for more specific ones.

As you know, I was looking for unique properties limited to specific cell types and not shared by others.

About β -glucuronidase, I have found that these reactive cells are morphologically typical of plasma cells. They have the same distribution as one would expect, and many of them are very strongly reactive. I have other data that substantiate this, but I cannot discuss it now.

Dr. Pearse: Where is the glucuronidase? May I just ask that?

Dr. Farnes: It is granular in the cytoplasm of the plasma cell, and it is usually around the perinuclear halo.¹

Dr. Pearse: It is not the ferric-hydroxyquinoline technique?

Dr. Farnes: No, it is the Hayashi technique.

Dr. Pearse: Fine.

Dr. Farnes: All right. No, I haven't ever used PMS for any of these things.

Dr. Pearse: I believe you should.

Dr. Farnes: I accept your suggestion gratefully, although my objectives have not been dependent upon the demonstration of very small amounts of dehydrogenases, and I didn't really put forth any isozyme hypothesis at all.

Dr. Pearse: You did in your paper.

Dr. Farnes: Whether it finally relates to histochemical specificity or not, I think it is appropriate to bring in any of these properties that may turn out in the future to influence our interpretation of these things.

Dr. Paul: I think we might take time now to discuss the whole question of the implications of histochemistry for cell tissue cultures. I am ready for comments and questions.

Dr. DeMars: Dr. Farnes has demonstrated something of real importance in the study of quantitative levels of enzymes in mass populations of diploid human cells. She has shown a single cell heterogeneity in alkaline phosphatase stainability, using naphthol MX phosphate.

Two laboratories recently have demonstrated that this heterogeneity is not a transient one: It is true that there is clonal heterogeneity from one cell to another in a more or less homogeneous population. Single cells can be isolated from these mixed populations, propagated, and grown to large clones which have either intense stainability or almost none at all. This was demonstrated in my own laboratory by Dr. Frank Walker, and (identically, I think) in Dr. George Martin's laboratory in Seattle. So Dr. Farnes' caution is very well taken. I would say the ability to make these clones also has created a very interesting phenomenon for tissue culturists to study genetically. But if these very marked differences in alkaline phosphatase levels reflect some aspect of the differentiated state of these cells, this is a trait of differentiation that persists through many cell generations.

Dr. Walker's work involved isolated single cells and growing them to populations of millions. These clones could be subcultured, although diploid cells are not supposed to be able to do that. I think this should be mentioned in connection with her work. I would like to comment that PMS will spice your G6PD tetrazolium stains tremendously. It really does work.

Dr. Paul: Thank you. Any other comments? Would you like to make any other point, Dr. Farnes?

Dr. Farnes: No. Thank you for your interesting remarks, Dr. DeMars.

Dr. Paul: Who is next? Histochemists?

Dr. Merchant: Not a histochemist. I might just say, in regard to the individual cell variation, that this also, of course, occurs in the heteroploid lines. We have reported studies recently in San Francisco, and are continuing them, in which we combine the variability in alkaline phosphatase with colony morphology as two independent markers which are extremely useful in following the changes in cell populations under differing environmental conditions.

We have situations in which we take "L" cells, growing in medium 199 supplemented with peptone and showing a marked heterogeneity with regard to alkaline phosphatase, and put these cells into Eagle's medium with no supplement. Within

¹ Since this meeting, Dr. L. T. Yam of the New England Center Hospital has informed me of work done by him and his colleagues substantiating the presence of β -glucuronidase in human plasma cells.

10-12 passages, we have selected from this population, cells that are only alkaline-phosphatase-negative. So far, in our hands, this is an irreversible process. We repeat: We have taken the same heterogeneous population, injected it back into an isologous host and grown a solid tumor, reisolated this in culture, and found that we have selected alkaline-phosphatase-positive cells. We have done this many times.

We have had other situations in which, going from a monolayer culture in the same medium to suspension culture and particularly to a high population density, we again selected for alkaline-phosphatase-negative cells. The alkaline phosphatase content, however, is not always associated in these different situations with this same colony type. There are many modifications, variations, and permutations of the relationship between colony type and the presence or absence of alkaline phosphatase. Interestingly enough, some clones of cells are very unstable with regard to alkaline phosphatase, and the colony will have positive and negative cells. And this will give rise, when it is recloned, to a heterogeneous population again.

Dr. Farnes: This is all very interesting. If there had been another enzyme or another histochemical property which I could have found to show the particular relationship in which I was interested, I would have chosen it. And I tried to be very careful in the time course that I used for interpretation. As I pointed out before, I used very short time intervals and watched the cells very carefully.

This enzyme, or group of enzymes, is probably one of the best studied, worst behaved enzymes in terms of its *in vitro* behavior. It may be induced in certain cell lines by steroids (glucocorticoids) and by substrates, in some. This is evidently an enzyme which is extraordinarily variable, and susceptible, as others have also shown, to a whole host of environmental factors—I am sure we have identified only a very few of them—and I wouldn't trust it any farther than I could see it, for perhaps 3 days.

Dr. Petrakis: I was very impressed with Dr. Farnes' histochemical approach, but I would like to change the emphasis and point out that the effect of tissue culture environment (no matter what it does to the enzymes) in a sense will be physiological, especially when we are dealing with fibroblast-like cells. Because if we look at the fibroblasts or the cells that presumably respond, say, to wound healing and enter the wound-healing cycle, this is a system that has probably evolved and been selected for many, many thousands of years.

And does tissue culture provide the environment that would promote any functional type of differentiation one might find *in vivo*? The reason I raise this (I am not going to talk about previous work) is that your discussion of the pokeweed mitogen-stimulating blastogenesis brings to mind some work we have been doing, trying to use wound fluid in place of phytohemagglutinin. We have been taking wound fluid from newly made wounds and using it as a stimulant in place of phytohemagglutinin. We find this has the ability to promote blastogenesis to a small degree, as indicated in a sense, by DNA labeling with thymidine.

I just wonder if the emphasis shouldn't be placed on trying to provide a more physiological environment to bring out the enzyme activities of these cells.

Finally, a histochemical test you didn't use was one for collagen or something at the terminal end of this reactivity.

Dr. Farnes: Are you referring primarily now to the blastogenic lymphocytes or—

Dr. Petrakis: In the blood, if you take blood, you can't really say what kind of cell you are dealing with, because, I suspect, if you take blood from the heart, you are going to get one kind of complex of numbers of cells; if you take it from the vein—a peripheral vein—you may get other cells. And when you make smears, they all look like lymphocytes and monocytes. I surmise that the only way one can define this is in the product the cells produce under specific environmental conditions. And I question whether tissue culture really provides this.

Dr. Farnes: Certainly, if I understand you correctly, I must agree with what you said. I hope I didn't give the impression that, in any of these systems I have been using, I imagined I had an ideal environment for differentiation of any sort, because I certainly don't. I would be as delighted as you if someone were to define a few of these factors more clearly.

Dr. Paul: We are trying.

Dr. Joachim: I would like to ask what substantiates the assumption that the lymphocyte can transform itself or can acquire phagocytic properties. I agree that most cells have phagocytic properties in cultures, but I thought the lymphocytes would be the last to be considered phagocytic cells. I know that this miraculous lymphocyte is now assumed to transform itself into almost anything, including plasma cells and macrophages, but I don't think there is enough evidence to substantiate this assumption.

Dr. Farnes: I hoped I had made this clear. This has been a debatable matter for a very long time about the progenitor cell in human blood cell cultures for the macrophages and multinucleated giant cells—specifically for the macrophages. I don't think the question can be considered entirely settled yet. My suggestion was made in hope that someone might become interested enough to do some ultrastructural studies following the evolution of these cells.

The extensive work by Rebusch's group, for example, using the skin window technique, gave some evidence which may be significant. I will grant this had the disadvantage that it is a serial morphologic kind of study at different time intervals, and that one can always claim there is room for confusion there, especially since the cell morphology changes so much during such a reaction. Berman's work has also substantiated this impression. Finally, there was a study, published last year from the laboratory of Dr. Israels, in which counts were done of the types of cells in the inoculum. The results suggested that the monocytes alone were not sufficient in number to account for the macrophage formation occurring in cultures in which no cell division was detectable.

Taking all of this together, I think that the probability of ultimately demonstrating this activity of the lymphocyte is really quite good.

Dr. Bonting: I have a question for Professor Pearse. I believe that biological research is most successful when it is quantitative, and he suggested that there are possibilities of making these histochemical tests quantitative. So I would like to ask what the possibilities are, and add two possible approaches I have seen recently that might offer some success.

One is the modification of the Kelly technique for cholinesterase whereby the crystal growth rate of the copper sulfate observed under the microscope and the timing of this formation of the crystal is used by Douglas as an indication of activity.

The other technique is the one developed by Ostrowski, in which he treats the enzyme, an esterase, or even adenosinetriphosphatase, with a competitive tritium-labeled inhibitor which then binds to the enzyme in 1:1 M ratio. The tritium autoradiography gives the quantitative measure of the local enzyme activity.

Dr. Pearse: Well, I don't think the second technique would appeal to everybody, even if its 1:1 ratio is actually proved, though I don't believe it is in most cases.

I think the other approach to the quantitation of the mass of the product is better. That is to say either by use of interferometry, as Danielli did many years ago when he estimated the calcium phosphate deposit from alkaline phosphatase activity, or by some kind of fluorometry. I think it has been and probably still is a promising approach.

The method based on the time of first appearance of the product is probably even better. All these methods are, of course, only semiquantitative or paraquantitative. If you want absolute quantitation, I think we are still a long way off

because the machinery for this seldom allows one to quantitate on a per cell basis, that is, per single cell basis. It only gives an average.

So I think that of all the techniques, probably the microassay techniques are still the best. But that is a counsel of perfection. For ordinary histochemists, probably something like the estimation of mass of the product or the timing of the first appearance of the product would give the best opportunities.

Dr. Moskowitz: In regard to the question of the environment relative to the appearance of alkaline phosphatase, we have been studying aggregation of cultured mammalian cells and have been working with a heteroploid monkey kidney line. This line does not produce any alkaline phosphatase in monolayer during a week of active growth. However, if we aggregate these cells, within 24 hours they stain intensely for alkaline phosphatase. So, taking them out of the monolayer environment and letting them interact with each other causes them to react with alkaline phosphatase.

Dr. Jacoby: I don't want to enter the discussion about lymphocyte-macrophage transformation because it is quite impossible. I only want to say that the Rebuck study has received a very severe blow by the work of Volkman and Gowans, which is perhaps more important than any other possible transition of the series done by Rebuck.

The main question I want to ask of Dr. Pearse concerns the peroxidase reaction of lymphocytes, which was just as amazing to me as perhaps the plasma cell story was amazing to him. The lymphocyte series is fundamentally peroxidase negative, which was known 100 years ago. What I would like to know from him is under what technical conditions he demonstrated the peroxidase reaction in lymphocytes, even if they were only a few granules?

Dr. Pearse: Of course, it is quite a gambit. We inject peroxidase into the animal, preferably in the organ or cell in which we are interested, and then see where it goes. I didn't believe the findings either. They amazed me because I was brought up to think that the lymphocyte was not a phagocytic cell. But it certainly takes up peroxidase extremely rapidly.

All the technique indicates is the same number and type of bodies (which I am going to call lysosomes before this audience, at least) as does the standard method for acid phosphatase. There is no peroxidase normally in the lymphocyte.

Dr. Jacoby: That's all right. That's all I want to know. Thank you.

Biochemical Taxonomic Enzymology of Certain Cell Strains *In Vitro*^{1, 2}

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SUMMARY

A study of the level of several enzyme activities of 14 long-term murine tissue culture cell strains has been made. The 14 strains had been adapted to continuous growth in chemically characterized medium with no added protein as a medium constituent. The source of 2 of these was the submaxillary parotid gland; the liver for 2; embryo mince for another; a mast cell tumor for a 6th; a hydrocarbon-induced lymphoma for a 7th; the lymphoid leukemia L1210 for the 8th; and the rest from connective tissue. The enzymes studied were: 1) arginase, because liver tissue in ureotelic animals is reputed to contain high activity of this enzyme and because arginase is a key enzyme in the terminal stage of the Krebs ornithine cycle; 2) amylase, because parotid tissue has the capacity to furnish this enzyme; 3) lactate dehydrogenase, to provide data on a dehydrogenase outside the citric acid cycle but feeding a product into it; 4) malate dehydrogenase, to include one enzyme within the tricarboxylic cycle;

5) catalase, because liver tissue contains much of this enzyme; 6) alkaline phosphatase, because reports in the literature indicate that high- and low-activity clones could be selected readily from human tissue in culture, and because liver tissue freshly excised is high in this activity; 7) acid phosphatase, because it is known that several cultured cell strains examined by others were high in this activity. All strains were found to have high acid phosphatase activity and all, low alkaline phosphate activity. No amylase activity was detected. Catalase activity was barely measurable even in the liver cells. Both lactate dehydrogenase and malate dehydrogenase were highly active in all strains. Arginase activity was present in all cell strains, but very low in all except one strain of liver origin, one strain from a sarcoma that on serum-containing medium had a very high activity. Moderate activity was found in one fibroblast-like strain.—*Nat Cancer Inst Monogr* 26: 229–246, 1967.

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

² Portions of this material were presented at the Miami Beach Meeting of the Tissue Culture Association, May, 1965. The author is spokesman in this report, which represents the collaborative efforts of many individuals over two decades.

³ National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

MY ORIGINAL thought with respect to today's discussion was to review in some detail the pertinent work in biochemical characterization of cells *in vitro* over the 10 years since the meeting in Woodstock, Vermont (1). The publication explosion makes such a review impossible in the time available. I would therefore remind you that some 4 years ago, the Tissue Culture Association held its Symposium on Analytic Cell Culture, the proceedings of which are now on record as NCI Monograph #7 (2). The following year the Symposium on Organ Culture was published as NCI Monograph #11 (3). A year later Monograph #13 (4) concerned with the problems of metabolic control mechanisms in animal cells was published. Last year's Symposium on the Chromosome held at the Miami Beach meeting of the Association has just been presented as volume 1, No. 1, of the new publication "In Vitro" (5). All these records have information valuable to any consideration of the characterization of cells or organs grown *in vitro*.

We can hope that the final goal of histochemistry and cytochemistry will be quantitative analysis of the cell and all its functioning parts. If this goal can be reached in the foreseeable future, it should add greatly to our parameters for characterizing the cells *in vitro*.

As part of a program for characterization of cells, we are now studying the behavior of certain enzymes as mammalian cells adapt to, and continue in, culture *in vitro* under various conditions. The study is predicated on the following hypotheses: 1) Enzymes mediate the exquisitely detailed metabolism of all living organisms. 2) Enzyme action depends on precise genetically determined structure. 3) The fitness of a chemical substance for its biologic function is not changed by growth *in vitro* or by the tumor process.

Oparin (6) in a recent review has suggested that the "origin of life should be regarded as an intrinsic part of the general orderly process of the development of the universe." Certain enzymes must have been there as the organized phenomenon we call life began.

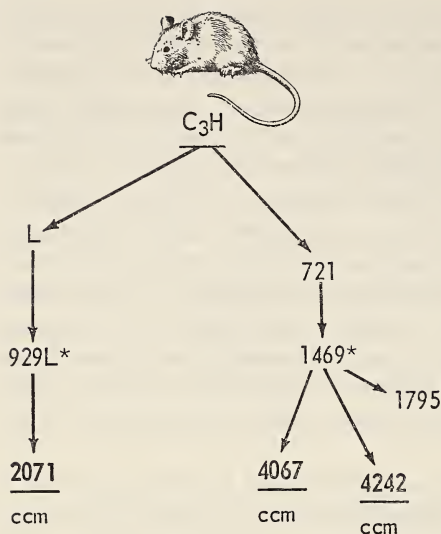
Cells grown *in vitro* offer a better than usual source of cells for the study of enzymes in general. This statement applies especially to the numerous cell strains now growing well in chemically characterized protein-free medium. You know that blood protein contains many enzymes which are a complicating factor in media. Much of this discussion will center around cells derived from the C3H mouse. (This is despite the fact that we have adapted to the characterized medium four cells strains of human origin: normal skin from a 65-year-old male (7, 8), bone from an embryo (9), the S-3 clone of the carcinoma strain HeLa (9), and the epidermoid carcinoma strain HEP-2 (9, 10). Also adapted are two other primate strains: kidney strain 3526 from the *Macaca* rhesus monkey and kidney strain 4952 from the green monkey (9). In addition, sarcoma strain 4206 from the Chinese hamster has been adapted to growth in this medium (11).

Please bear in mind that the primary charge to us, as public servants in the laboratory, is to study transformation of normal cells to the malignant state. The C3H mouse strain cells were chosen for our main original effort for several reasons: 1) The C3H strain of mice was at that time the most highly inbred one readily available in quantity. It had been carried for 60 generations by mating littermate male to littermate female so that the population was genetically homogeneous. 2) During these 60 generations it had been monitored for cancer incidence, and it was known that spontaneous sarcomas occurred only rarely in the C3H strain. Since connective tissue cells were to be cultivated, this attribute seemed desirable. 3) Mice are relatively inexpensive to grow and maintain for the long periods needed to learn whether the cultured cell would give rise to tumors on return to the animal. Cost was a factor to be taken into account. NIH has not always had a big budget, and even today does not have it for intramural research.

Our choice of an inbred animal can be criticized on the basis that it does not very well represent the randomness of what goes on in nature among wild strains, including the human where mating is random. Mice of the C3H strain, perhaps as a result of the close inbreeding, manifest certain peculiarities such as congenital blindness.

It was from one of this strain of mice that the now ubiquitous sarcoma (text-fig. 1) tissue culture cell strain 929L developed. Also the three fibroblast strains from the single clone extensively studied by Dr. Katherine Sanford (12-14) and used by Drs. Barski (15, 16) and Spencer *et al.* (17) for hybridization of cells *in vitro* are from this strain of mice. These three fibroblast cell strains are the so-called "high tumor" strain and the two "low tumor" strains. They are *not* substrains of 929L. Their only relationship to L is that they were taken from the C3H strain of mice 60 mouse generations after L was originated, and L in the meantime had undergone 3,000 replication generations *in vitro*. The separation in time is such that the equivalent in terms of the human experience for 60 generations is more years than have elapsed since William slew Harold at Hastings (18). I belabor this point because the very comprehensive volume 1 of the series being edited by Professor Willmer (19) has the three strains confused with L. When a confusing statement such as that gets into print in what bids fair to be the authoritative text on the subject, it tends to be perpetuated.

When I first joined the NIH laboratory years ago, two of the interesting cell strains under study were the fibroblastic cell strain L and strain 721 derived from the liver epithelium of a day-old mouse. Both of these were from the C3H strain of mice and there was never question as to their being mouse cells. Their morphology, however, was very different. Looking for some definitive biochemical difference, we examined such properties as glycogen formation and storage: 721 cells readily stored glycogen



TEXT-FIGURE 1.—Origin of the fibroblast strain 2071 and the liver strains 4067 and 4242 on chemically characterized medium.

and then mobilized it as the glucose of the medium was depleted. *In vivo* liver cells of the mouse have a very high catalase activity, while connective tissue has practically none. The L cells had barely detectable catalase activity, the 721 cells three times as much, but only a fraction of 1% of that of freshly excised liver tissue. Catalase is now known to have a half-life of 24 hours (20). Apparently under cultural conditions there is little need for maintaining even moderate catalase activity, so that the high activity of the tissue directly from the animal was quickly lost. Both cell strains had high acid phosphatase activity; both had low alkaline phosphatase activity; both had high lactate dehydrogenase and malate dehydrogenase activity. The arginase activity of the liver strain was comparable to that of liver tissue directly from the animal; that of 929L only one-fourth of 1% that of 721.

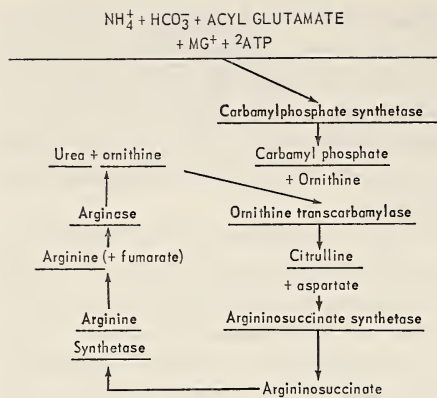
Since the enzymes of the complete Krebs-Hensleit ornithine cycle were reputed to be confined to the liver in the mouse, it was originally hoped that possession of the enzymes by the 721 strain of tissue culture cells would provide supportive evidence as to the tissue of origin. Exploratory studies when the cells had been in culture 3 years indicated that, except for arginase, the 5 enzymes were present at a level, even lower than in the liver of the C3H mouse strain of origin. Methods for the first 4 enzymes were poorly developed for the quantities of tissue available and the inquiry was not continued except for arginase itself. The clonal strain 1469 was taken from 721 a year later. Six years after cloning, the strain 1469 was examined for the 5 cycle enzymes in collaborative work with Dr. G. W. Brown, Jr., and Dr. P. P. Cohen (21). At that time all except the two terminal activities, *i.e.*, the cleavage enzyme and arginase itself,

were below detectable levels. The clonal strain had the four steroid reductases found in liver, though at a low level of activity. The activity level of these four reductases is known to be under genetic control and is lower in the liver of the C3H mouse than in most laboratory strains. Two years after cloning, 1469 gave two tumors from 142 injections of the cultured cells. These tumors rising at the site of injection were diagnosed by the pathologist as hepatomas (text-fig. 1).

Most of what I have just recited to you is already in the archives as ancient history. I recapitulate it here only to emphasize that not one alone but all these parameters were needed to identify the 1469 strain cells as of liver parenchymal epithelial origin. The two hepatomas on subinoculation into the animals gave 100% tumors. From one of the original hepatomas we have the long-term strain 1795 now in culture 12 years. (*In vitro* this hepatoma strain no longer stores glycogen but, like its ancestors, retains the high arginase activity.) Other reputed functions of the two original strains referred to have been examined. Fibroblasts are reputed to form collagen, but to date I have been unable to demonstrate that the strain 929L or any of the other strains we have derived from connective tissue or fat pads do so in the sense of forming it and excreting it into the medium.

The enzyme arginase is known to have a half-life of about 4½ days (22). Why the three strains of liver origin retain their high arginase is puzzling. This enzyme is the catalyst in the conversion of arginine to urea and ornithine, a function presumed to take place in all ureotelic animals. We have found little evidence that it functions this way in the cultured cells. Our medium is about 0.16 μM in arginine, but neither the 721 cells nor 929 cells convert as much as 1% of this to urea. The arginine is apparently used to make protein and not to supply energy. We have found arginase activity in practically all tissues of the C3H mouse (*cf.* 23). It has been suggested that such ubiquity must be associated with some role other than that of its mediator function in the Krebs cycle. A possible role in arginine transport has been postulated, but no firm evidence obtained to support this proposed function. Cedrangolo and co-workers (24) have raised this question of urea formation by routes other than the Krebs cycle enzymes using the intact rat as subject. However, no firm evidence has been obtained to support this suggestion. Text-figure 2 shows the intact Krebs ornithine cycle and the place of arginase in it. If a lesion occurs in any enzyme, the consequences to the host animal are usually serious. However, the 1469 cells survive and grow with levels of the first three below detectable levels.

Findings in the human diseases citrullinuria (25) and argininosuccinuria (26), which lead to mental retardation of the individual with essentially normal urea excretion, point directly to the fact that we must increase our knowledge of certain physiological aspects of the behavior of arginine and urea. The citrullinuria and argininosuccinuria are known to be under genetic control, with an autosomal lesion found on chromosomal



TEXT-FIGURE 2.—Krebs ornithine cycle for the formation of urea.

analysis is one patient (27). Another genetic disease of the human involving the Krebs cycle enzymes has been found (28) in which blood urea is normal but ammonia and glutamine are high, with the liver ornithine transcarbamylase only 20% of normal.

I mention these three human diseases briefly to illustrate the fact that as knowledge of enzyme patterns increases we may expect to find disturbances similar to these on rare occasions in our cells in culture. Much more serious, as a practical problem, is the fact that there is an alternate pathway for the degradation of arginine used by bacteria and first extensively studied by Hills (29) in 1940 with streptococci. This degradation is mediated by the enzyme arginine dihydrolase or deiminase to give ornithine, CO_2 and NH_3 . This enzyme is possessed by the filter-passing mycoplasma, pleuropneumonia-like organisms (PPLO). It must have been the presence of mycoplasma that led to some reports in the literature that addition of arginine alone to apparently exhausted media restored cell growth (30, 31). Some contaminations were traced to infected calf serum (32-40), and this, though put through the usual bacteria-excluding filter, passed infection to the cells. An increase in ornithine concentration in the used medium from growths of mammalian cells has to be taken as pathognomonic of PPLO infection.

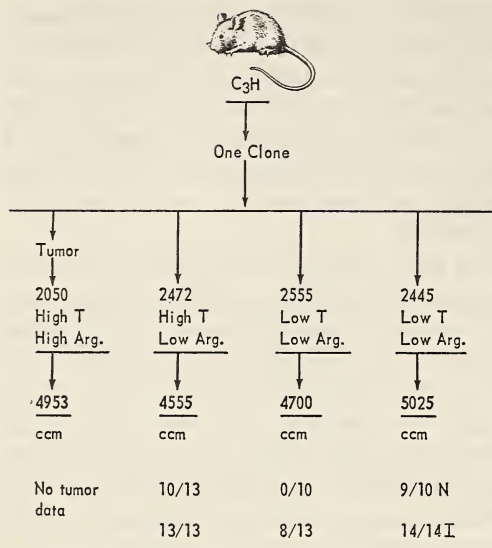
Catalase activity of the 721 strain is less than 1% that of the freshly excised liver tissue, and the arginase activity remains much the same as that of the liver. Such findings emphasize the fact that where there is change in one enzyme activity it can occur independently of the other enzyme activities of a given cell. As Florence Moog has pointed out, enzyme activity should be examined on a per cell basis to indicate whether a change either up or down is simply due to cell selection (41). We must remember constantly that, when we start cultures, we may from the beginning be selecting for cells that survive well, using glucose metabolically rather than glucose plus fat, as in the animal. Changes in enzyme

activity of the cell could reasonably be expected to influence cell metabolism, but our ignorance is still too vast for us to predict in what way. Hence we feel the need to have histochemical studies of enzymes on the individual cells as well as biochemical studies on the extracts in which cells have been disrupted and for which the population had been measured. I am therefore encouraged by seeing younger individuals such as Dr. Farnes enthusiastically working in the field of histochemistry, and I hold that such effort is to be abetted and applauded.

I present the story on 929L and 1469 mainly as background for some discussion of the three strains descended from them growing on chemically characterized medium. The high-sarcoma-producing substrain 2071 grows well in the absence of added protein in the medium. You will recall that 2071 was one of several lines from 929L "adapted" to growth under these circumstances, but this is the only one continued. Substrain 4242 also grows well and by latest test has higher hepatoma-producing capacity than its clonal parent. Its protein content both on a wet-weight basis and a per-cell basis is only about 60% that of its parent. Its arginase activity is less than 5% that of its parent. I take this as one indication that, under the more rigorous conditions imposed by growth in the characterized medium, unneeded protein may not be maintained at a high level. It is known that in bacteria, under appropriate circumstances, a single enzyme may constitute as much as 10% of the total cell protein (42). In contrast, to arginase activity, acid phosphatase activity per cell remains the same as for the parent. On a total protein basis it appears higher, reflecting the decrease in total protein of the cell. The dehydrogenases for lactate and malate behave in similar fashion, both maintaining high activity. The 4067 substrain has arginase activity some four times that of 4242, but grows much less luxuriantly. The 4067 cells are among the more fragile cells under study.

From the high-tumor-producing strain of fibroblasts and the three low-tumor-producing strains 2472, 2555, and 2445 already referred to, we now have the derived strains in chemically characterized medium shown in text-figure 3.

These cells from 2472, 2555, and 2445 had a common origin as lines from a clonal strain, as already documented (14, 43). They all had barely detectable arginase activity as did their parent 15 years ago. The strain 2050 which previously (43) had 400 units of arginase activity per 100 g protein had by the process of adaptation to medium 135 decreased to 20% of its former value, behavior similar to that of 4067 and 4242 referred to above. The lactate dehydrogenase activity was high for all four strains, likewise malate dehydrogenase and acid phosphatase. All had barely detectable alkaline phosphatase activity. Many of the rodent strains have low alkaline phosphatase activity, but beyond this one may not generalize, because there are exceptions. The L1210 (44) murine lymphoid leukemia



TEXT-FIGURE 3.—Origin of high and low tumor strains now in chemically characterized medium. Strain 2050 differs from the others in originating from a tumor from cells of the original clone.

strain 5439 from the DBA strain of mice carried on medium 135 plus 10% horse serum in suspension culture has the greatest alkaline phosphatase activity of any of the strains in the laboratory. The six primate strains on the chemically characterized medium all have considerable alkaline phosphatase activity, but 5439 is much higher in activity than these.

In addition to the strains mentioned so far on the characterized medium, we have strain 3681, fibroblast-like in appearance, from minced mouse embryo taken into serum-containing medium, then into medium 109, and now on medium 135, low in alkaline phosphatase, high in acid phosphatase activity, high in lactate and malate dehydrogenases, and intermediate in arginase. Next to the 4953 strain from the high tumor-high arginase strain 2050 mentioned previously, this strain 3681 has the highest arginase activity of the fibroblast-like strains on the characterized medium. Strain 3681 is now 6 years from origin. Very similar, but without a relatively high arginase activity, is the strain 4705 (45) but, unlike 3681, it was started as fresh tissue directly in the chemically characterized medium some 3 years later than 3681. In the enzyme pattern so far explored, 4705 is much like 3681, except that the arginase activity like that of 2071 is much lower. Another strain studied is 4075, from the submaxillary parotid tissue of the Swiss mouse, now a tumor-producing strain. A clonal strain, 4075 closely resembles 4705. I was unable to find any amylase activity either in these cells or in the used medium from their cultivation. The other enzyme activities were much like those of strain 2071. Another clonal strain from parotid tissue, NCTC 3954 (9), was indistinguishable enzymatically from 4075 except that it had a slightly higher

arginase activity. Current procedures for measuring amylase activity under these circumstances need improvement.

The strain P-815 mast cell of Dawe and Potter (46) from the DBA/2 mouse has been adapted to growth in medium 135 under the NCTC strain number 5223. This strain grows luxuriantly in culture in roller tubes. Enzymatically, as far as examined, it resembles the fibroblasts. The strain 3749 derived from a methycholanthrene-induced lymphoma in the DBA/2 mouse had an enzyme pattern, so far as it was examined, much like that of the fibroblast strains, except that the alkaline phosphatase activity was greater. The lymphoid leukemia strain 4972 from the same strain of mouse was similar, but with still higher alkaline phosphatase.

Table 1 gives data on the origin of the above-mentioned murine cell strains.

Work on possible isozyme patterns in the cells as they grow in different media, especially as they adapt to growth in characterized medium, has barely begun (47). Much work needs to be done on enzyme patterns in these cells, particularly in such areas as lactate dehydrogenase isozyme activity where there is so much interest in studies collateral to tissue culture (47-63), glucose-6-phosphate dehydrogenase isozyme patterns as possible

TABLE 1.—Origin of certain murine cell strains referred to in text

NCTC strain No.	Original cell strain	Years in culture in 135*	Density of growth	Present status in tumor production
3681.....	C3H mouse fibroblast.	6	Taken into 135 + HS and then into 135 alone.	High sarcoma strain
4705.....	Minceed embryo C3H mouse.	3	Directly in 135.....	High sarcoma strain
4075†.....	Parotid tissue Swiss mouse.	4	In HS + 135 and then to 135 alone.	Parotid tumor strain
3954†.....	Parotid tissue C3H mouse.	5	In HS + 135 and then to 135 alone.	Parotid tumor strain
5223.....	Mast cell DBA/2 mouse.	1	In 135 after 135 + serum.	P-815 lymphoma
3749.....	DBA/2 mouse...	6	In 135 after 135 + serum.	MCA‡-induced lymphoma
4972§.....	DBA mouse strain L1210.	2	In 135 after 135 + serum.	Lymphoid leukemia strain

* 135 = NCTC characterized medium 135 = 109 - cysteine.

† Clonal strains.

‡ MCA = methycholanthrene.

§ 4972 on NCTC 135 is from the strain 5439 referred to in the text as growing in 135 + 10 horse serum in roller tubes showing high alkaline phosphatase. In NCTC 135, activity is intermediate in range.

aids in strain identification within a species (64), and the various esterases found to be present in mouse tissue (65, 66).

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DISCUSSION

Dr. Paul: Would Dr. Chang care to comment on this paper?

Dr. Chang: I suspect that both Dr. Paul and Dr. Westfall don't like the term "taxonomic enzymology." It may be worthwhile to briefly examine this term for its exact meaning. I think Dr. Ann Cohen has best defined taxonomy in her article published in the *Journal of General Microbiology*, 1964. She defined taxonomy as the art of classification, nomenclature, and identification. Referring to a particular case here, in a histonomic sense, enzymology of cell culture, I think what Dr. Westfall is trying to determine is whether, by studying the enzyme profile of different cells, he can group cells according to certain similarity in the enzymatic profile and to determine whether cells belonging to certain groups may have some interesting characteristic.

Two questions especially important to us probably are: whether cells in mammalian form include any special enzymatic pattern; whether the cell from a certain tissue origin may have a certain enzymatic pattern.

I think that the results Dr. Westfall has presented to us so far appear to be very disappointing because I think he is putting a tremendous amount of work into it and showing that almost all cells—malignant or nonmalignant, from monkey or mouse, or even from the liver or fibroblasts—more or less have the same type of enzymatic profile. But I don't believe this is not important work; I think this is tremendously important work. I think this is work that must be done, and hopefully, Dr. Westfall will continue to do it. Hopefully, he may discover some major difference in, for instance, the malignant cell as against the normal cell.

I think some of you may ask yourselves, "Why spend all this time in doing an experiment and study like this when you can easily test malignancy by inoculating cells directly into the animal?" Or, you can say, "Well, cells still retain certain characteristics of the tissue of origin." If you again inoculate the cell back to the animal, they will differentiate again.

But don't forget a lot of us are drawn to the tissue culture field because tissue culture provides us a means of studying living human cells, you see, and we just can't do that. If somebody can show us a way to tell whether a human cell in tissue

culture is normal, malignant, and so forth, I think he has made a tremendous contribution.

Dr. German: My comments are brief and only an extension and perhaps an addendum to the papers of Dr. Farnes and Dr. Westfall. My point, already made by several discussants at the end of the first paper, was that a cell or cell type may change its characteristics in culture, and the taxonomist must remember this. There are many examples. For instance, diploid cells in the lymphocyte change dramatically when cultured with and without phytohemagglutinin. Then, consider the studies of Volt, showing that aneuploid cells in a highly selected medium may change their vigor. A more vigorous line which can grow in the new medium is selected for. Not only that, but there is a genetic change—the modal chromosome number of the cell line shifts.

Other examples of change are shown in the experiments of Vessel, Bearn, and Philips, using embryonic chick and embryonic rabbit. They demonstrated that the LDH isozyme patterns changed at various stages of embryonic development. They showed that 10-day chick embryos in tissue culture underwent rapid, within-48-hour change in the isozyme pattern, with a shift from predominantly LDH-1 to LDH-5.

You will remember that the LDH isozymes are composed of two subunits, subunit A and subunit B, each apparently under a separate structural gene. The production of LDH-1, consisting of four B subunits, rapidly changed and then continued to shift. After a few days, the predominant isozyme was LDH-5, consisting of four A subunits. Most long-term-culture cell lines show a predominant LDH-5 pattern, so that in almost all laboratories, under the various culture conditions used, this has been the type isozyme selected for.

We were surprised to find that this was not the pattern when we attempted to characterize some of the National Cancer Institute's cell lines, grown for many years in chemically characterized media without serum supplement. These lines showed a different pattern, including a clonal subline of HeLa—HeLa S-3. In these lines, there was a predominant LDH-1 and essentially no LDH-5. The first guess, of course, was that these rigorous cultural environments, for many years without access to serum or other proteins, had selected for a line which made predominantly subunits B and predominantly, therefore, LDH-1. Interestingly enough, addition of serum to the medium promptly caused a shift to the left. The question, then, was: Is this genetic induction or genetic selection? Was there a new cell type selected by the addition of serum?

We have been unable to clone these lines, but by timing experiments—that is, adding serum and then inspecting the isozyme pattern at various periods afterwards—we have been able to show that certainly within 18 hours and probably within 6 or 3 or even 1 hour—certainly less than the cell generation time—there is a shift to the left of the isozyme pattern. This probably rules out selection, but there are various other mechanisms, the most appealing of which is an actual change in the activation, either transcription or translation, of the relative increase in activity or relative decrease in activity of either the A or the B structural gene. I use this only as an example to show that the same cells under different *in vivo* or *in vitro* conditions may change their enzyme characteristics.

Dr. Rothberg: I wonder if Dr. Westfall meant, in relation to arginase and the urea cycle, to leave the impression that your epidermal cells had the full urea cycle or only arginase?

Dr. Westfall: You mean human epidermis?

Dr. Rothberg: Yes, or your epidermal cell lines.

Dr. Westfall: We haven't checked the others. This is just arginase.

Dr. Rothberg: Well, in the past, I have examined whole epidermis, and strangely enough, arginase is always present, but the entire cycle doesn't exist. This is of

great interest, because George Rogers in Australia has shown that wool follicles do have the entire cycle. So it is possibly a question of how rapidly your epidermal cells are keratinizing. I think we know, for instance, that we do get exaggerated arginase activity when hyperkeratinization occurs.

Dr. Westfall: Thank you.

Dr. Schimke: I just want to get something clear about the urea cycle. The full urea cycle has been shown to exist only in the liver. The enzymes that don't exist in other tissues are the first few. Carbamylphosphate synthetase may occur in a slight amount in intestinal epithelium, but certainly the other enzymes (ornithine transcarbamylase, argininosuccinate, synthetase, arginine synthetase) are primarily only liver enzymes. The others exist in all tissue.

Dr. Westfall: The point is well taken. As I understand the term, "ureotelic" means that the system under study must be able to carry out the primary "fixation" of CO_2 and NH_3 to yield carbamylphosphate and have the other enzymes you list in order to form the terminal urea.

Dr. Coriell: I hope the test Dr. Westfall described will work out to help us distinguish between human lines. This is really a thing that the Cell Culture Collection has needed. The collection started because contamination of cells became apparent in the late '50's, *i.e.*, contamination of the bacteria, viruses, mold, weed, fungi. Then, some of the rabbit cells and other cells turned out to be human cells. It was assumed that some of the human cells might also be contaminated with HeLa, but there was no way to prove it. Now, if this can be proved and added to the parameters by which the ATCC screens, many of these recurrent problems can be eliminated. I am positive they have all been eliminated in those cell lines in the Collection. There is still a glaring gap in our knowledge of how to identify contamination for sure. I would only reiterate what Dr. Pearce said: Where two tests are possible, don't be satisfied with one. I understand that here twelve tests are available—eleven in addition to glucose-6-phosphate dehydrogenase. And so, to make sweeping claims on the basis of only one test as Dr. Gartler did seems to me foolhardy, perhaps. I would ask Dr. Gartler to apply these other tests, because I hope that you can really fingerprint cells and I believe it is perfectly possible we can fingerprint cells and people's sera eventually when we know enough, because there seem to be great differences in individual serums and in these enzymes. So I think it probably can be fingerprinted. But, doing it on the basis of one, reminds me a little bit of the three blind men examining the elephant. Examination of a partial area can be very misleading.

So I hope people will not go away from here thinking that this one test does settle the problem that they are all contaminated. And there are other suggestions by Dr. Gartler that they are heterozygous, many people are heterozygous, for A and B, and the kinks are out of the culture. So with just those two things alone, we have to be very cautious about it.

Dr. Chang: I would like to add a comment to the discussion of arginase. I think we all too often think of the arginase as only part of the urea cycle. We know that the polyamines, putrescine and its derivatives, spermadine and spermine, are present in ribosomes of all leading organisms. Unfortunately, the metabolism of putrescine, as far as genesis of higher animal cells is concerned, is not clearly known. Conceivably arginase could give rise to ornithine, which could subsequently give rise to spermadine and then putrescine. I think Lieberman has shown that putrescine is required in some cell lines. In many of the other cells where the Krebs urea cycle doesn't seem to play a role the arginase could conceivably influence the mode of formation of putrescine which, of course, is present in all animal cells.

Dr. Ioachim: I would like to make a plea for morphology. I think that this meeting has shown morphology to be really neglected. We are at the end of the second day. We have seen innumerable curves, but only this evening have we seen

the first cells projected on that screen. It is my understanding that somebody who really looks at his cultures day after day wouldn't experience such miraculous changing from one strain to another, from rabbit to human as has been reported in previous years.

I think there still are some morphological differences, and I also think when we speak about changes in enzymatic pattern, it would be desirable to show some cultures demonstrating the way they look in their transformed appearance.

Dr. Paul: Dr. Westfall, you probably know quite a bit about the morphology of your cells.

Dr. Westfall: In our laboratory, I seldom look at the cells, but the biologists do constantly, every day. And they keep a photographic record, in addition, as the cells are carried along. We do attach importance to that.

I don't know whether Dr. Sanford, for instance, would want to add anything to this.

Dr. Pearse: I doubt whether the light microscope is sufficiently accurate. I think you have to use the phase microscope.

Dr. Paul: We have a spherical L cell, just by the way. It is an L cell.

Dr. Dickson: I should like to ask Dr. Chang a question. For how long do liver cells *in vitro* retain tissue-specific antigen? Secondly, I should like to ask the panel: To what extent can a tissue-specific antigen be used to characterize cells *in vitro*?

Dr. Chang: I don't know the answer to your question. I never started that. I think this is a good time to emphasize the point that the line was named liver cells. I don't know by whom. All I have ever done was publish a paper in 1954 saying a cell strain had been derived from a piece of biopsied liver tissue. And I do not believe that the cells, after multiplication for so long, still retained this specialized function.

Dr. Paul: Do we have any immunologist who could comment on the last point? I think this could be a useful bit of information.

Dr. Friedrich Deinhardt: I cannot answer the question for you, but the man who could, unfortunately is not here. He is Dr. Bryant from Minnesota, who has recently published on this and who is working, not only on tissue-specific antigens, but on transplantation antigens in these cells. At least, we have talked with him. And among some of the cells which were shown on the board this morning, he would say there are some definite differences antigenically. His study has gone on for many years, and I am sure more detailed accounts will come from Dr. Bryant in a short while.

Dr. Brown: I would like to report on some work with which I am cooperating at Walter Reed Army Institute of Research. It has to do with the culture in suspension form of the L strain and growth to a stationary condition in which the cells are maintained at about 6-8 million cells per ml for a month or 6 weeks. Under these conditions, we find a production of hydroxyproline characteristic of collagen. We also find a tremendous increase in the lactate dehydrogenase enzyme activity, indicating that under differing conditions, you will find the same cell line is producing different things. These cells, when returned to static dilute culture, perform in a characteristic manner.

Dr. Merchant: I think the answer to this question is undoubtedly "yes" in some instances and "no" in others. I gave my ideas about this at San Francisco in some detail. I think when we come down to the level of tissue specificity we are beginning to deal with the kinds of things that are likely to be more evanescent than those which contribute to species specificity. I feel that species antigens are a result. They are primarily materials or cell components that are necessarily related to the structure because they have survived long periods of evolutionary change. When we come to the tissue-specific antigens, we are starting with the fertilized ova, with a cell

that has the information for all of the things that are in the host. Which ones are expressed and which ones are not expressed depend upon the differentiation of the tissue. And sometimes, these things are going to be, that we get out and look at as being tissue-specific—tissue-specific because they represent enzymes that are specific to that tissue. In some cases, they are going to be products of enzyme action. There are probably a host of other possibilities. There are some, such as changes in structure, where you have ciliated cells, where you certainly are going to have a much more permanent situation.

So I think there is no across-the-board answer, but there are certainly going to be many instances where we can find tissue-specific antigens.

I might say that in our laboratory Dr. Longren has evidence for antigenic differences between some of the cells that were listed as probably all being HeLa. We have not been able to show any antigenic differences as yet between HeLa cells obtained from a number of different laboratories grown under quite different conditions, some of them that have certainly been selected quite differently, and yet we see differences, antigenic differences, between some of these cell strains.

Dr. Paul: Thank you. I think, obviously, we will want to find out who "C" and "I" actually were in that table this morning.

Dr. Amos: Dr. Pearce, I think, referred to cytolysis, talking about the uptake of peroxidase, soluble protein. Some of us who are interested in the uptake of large molecules can't let him get away with that.

I think Dr. Jacoby also.

Do you call this phagocytosis?

Dr. Pearce: Very, very badly and very wrongly, I suppose. I just meant to take up a molecule.

Dr. Paul: He just meant the uptake of molecules.

Dr. Amos: I think on that basis, so far, one can't distinguish too much between cell types. It turns out that many of them take up large molecules—nucleic acids, at least, and proteins. And it depends more on some property of the molecule than some property of the cell.

Dr. Pearce: Yes, but you could ring in an immense number of changes if you practice enough and finally make some accurate decisions. You can do a lot of work, I think.

Dr. Kite: I just wanted to make one comment on the question of tissue-specific antigens because we, too, have been very much interested in this. In studies of thyroid and thyroiditis, one may find that in human thyroid or rhesus monkey thyroid, there is an organ-specific antigen in the cells of the follicle or the cells lining the follicle and that this organ-specific antigen may be detected by immunofluorescence, by cytotoxicity.

And we have studied these cells in culture and, both by cytotoxicity and immunofluorescence as well as mixed agglutination, have found that this organ-specific antigen can be detected by immunofluorescence, for as long as a week. This is a very fleeting antigen, and to the best of our abilities with the various environmental situations, we have been unable so far to find the conditions under which it will persist for a longer time. I think the thyroid is such a very good example, very good model system, because of this very nicely demonstrated organ-specific antigen, that we are anxious to see if we can find ways of getting it to persist longer.

Dr. Pious: I would like to comment on the possibilities of antigenic identification of cell strains, since this is a most promising area in which to increase the panel of markers by which we can distinguish intraspecific cell strains from individuals within a species. A number of investigators have shown over the past 10 years that various mouse cell lines have retained the homograft reactions of the donor strain.

In our laboratory, we have worked with cultured diploid rabbit strains and have shown that they maintain a consistent panel of reactivity against the panel of antisera.

It remains now to identify antisera which recognize human cell strains, despite theoretic difficulties. There are practical problems, but it should be possible to recognize strains derived from different human individuals.

CELL INTERACTION AND ORGANOTYPIC EXPRESSION

Chairman: Dr. Honor B. Fell, Strangeways Research Laboratory, Cambridge, England

Speakers: Professor Michael Abercrombie, University College, London, England
Dr. Clifford Grobstein, University of California, San Diego, California

Panelists: Dr. James Lash, University of Pennsylvania, Philadelphia, Pennsylvania
Dr. Aron Moscona, University of Chicago, Chicago, Illinois
Dr. Edgar Zwilling, Brandeis University, Waltham, Massachusetts

Dr. Fell: Ten years ago, at the First Decennial Conference at Woodstock, I took the Chair at a session on tissue culture in relation to morphology. Under pressure from the management, I was rash enough to make introductory remarks about the future of tissue culture in this field. The other day, I turned up my comments on this occasion to see how good a prophet I had been. I said, "I believe we are entering an era of what we may call physiological morphology." I am sure you will agree this has proved true. The various lines of work that seemed particularly promising at that time were the experimental analyses of intercellular relationships. And, indeed, tremendous progress has been made since the last Decennial Conference. The latest views and ideas about this very exciting subject are presented and discussed by a group of leading experts in the field.

Contact Inhibition: The Phenomenon and Its Biological Implications¹

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SUMMARY

Orientated cell movements occur both *in vitro* and *in vivo*. Their analysis *in vitro* suggests that four main behavior mechanisms may be involved: contact guidance, chemotaxis, contact inhibition, and associative movement. In this review, contact inhibition is discussed at length, with some consideration of its relation to associative movement. Contact inhibition clearly varies in intensity according to the types of cell that are reacting and their environment at the time. There are probably specificities in the mutual reaction, but in general it seems that malignant cells and leukocytes are relatively deficient in inhibition by a range of nor-

mal fibroblasts. The effect of conditions, especially of the nature of the substrate, allows some analysis of the mechanisms involved and indicates that differential adhesion to cells and substrate plays a part. Associative movement, the drawing together of cells that have made contact, has a particularly important manifestation *in vitro*, in the phenomenon of sorting out of mixed aggregates, which also evidently depends on differential adhesion. The evidence that either of these mechanisms takes part in the morphogenetic processes of whole organisms is limited but not discouraging.—*Nat Cancer Inst Monogr* 26: 249–277, 1967.

IN THE whole animal, during periods of morphogenesis, cells often undertake directed migrations of many times their own length; and innumerable smaller scale displacements, active and directed, are likely to be responsible for setting up the finer details of cellular arrangement within tissues. Active cell movement, that is to say movement which is not merely the result of growth pressures cooperatively generated by a population of cells, is in fact profoundly involved in animal morphogenesis; and when, as in malignancy, it goes wrong, it as effectively disrupts the form achieved.

Populations of cells in tissue culture, dissociated from their original tissue structure either artificially or through their own locomotory activities, also show orientation of their movements, and as a result they too take up characteristic spatial patterns. They do not necessarily do the

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

same things they do *in vivo*. They may sort themselves out according to tissue type in mixed reaggregates, or take up a circular colony form when emigrating from a tissue fragment on a substrate, which are things they may never be called on to do in the organism. But the underlying mechanisms of their directed displacements in culture are open to analysis with a facility that is altogether lacking for the analogous displacements in the organism. A picture that is beginning to have some coherence is building up of the control mechanisms of locomotion in tissue culture environments. We can be confident that the tissue culture environments do not yet comprehend enough of the relevant variables to allow one to treat what happens in the organism as a predictable outcome of a general theory of cell movement, but this of course is the goal.

BEHAVIORAL REACTIONS *IN VITRO*

Four main control mechanisms for the orientation of cell movement appear to be at work *in vitro*. They are *contact guidance*, *chemotaxis*, *contact inhibition*, and *associative movement*. (I am leaving out of account those mechanisms which only affect speed of movement, since the importance for morphogenesis within the organism of the temporal interlocking of the various cell movements is little known.) Some of the four mechanisms proposed may in the end turn out to be varieties of each other; but at present they seem to be sufficiently distinct categories for the purposes of analysis.

Contact guidance, the orientation of cell movement according to the orientated structure of the substrate, has historical roots going back to the beginning of tissue culture. Analysis of how it works clearly awaits some advance in knowledge of how cells move, which is the greatest gap in cell physiology. Is the substrate structure in some way imprinted on the cell, or are competitive relations between "pseudopodia," *i.e.*, between subdivisions of the locomotory machinery, involved (1)? But in spite of this block to a penetrating analysis, which afflicts all behavioral studies on cell locomotion at present, and because of the great role often assigned to this mechanism in the organism, there still seems much to be done at a behavioristic level in surveying the extent to which contact guidance occurs in different cell types in different conditions. I have had no systematic experience with this form of behavior, so I shall not attempt to discuss it further.

Chemotaxis is a mechanism that is only rarely demonstrable as an important aspect of cell behavior. It has a primitive form, an example of which Weiss and Scott (2) have ingeniously constructed, which may be called "boundary chemotaxis." This merely requires that, when any part of a cell lies in a region with a higher than critical level of a certain environmental constituent, the locomotory machinery within that part of

the cell shall be paralyzed. If half of a cell is in a region above the threshold, while the other half lies across the threshold boundary, the cell, if it moves, can only move away from the inhibitory region. The mechanism will shift a cell no more than one cell length in a direction perpendicular to the boundary, unless the boundary itself shifts. It may, however, suffice for the short-range spacing out, by mutually repulsive chemotaxis, that leukocytes seem to show (3), and perhaps for the similar behavior that Twitty and Niu demonstrated in cultured embryonic melanoblasts (4). The more complex form of chemotaxis, which may be called "gradient chemotaxis" requires the cell to respond to a gradient of concentration rather than to an absolute value; there must be some form of intracellular coordination to compare concentrations at opposite ends of the cell, and only granulocytes and macrophages have been shown to be able to do it (5). With them, it may be a very specialized mechanism (6). It does not seem that chemotaxis can be a dominant form of behavior in most cells in culture, and I shall not consider it further.

Of the remaining two mechanisms, my own familiarity with the process and the extreme conspicuousness of its results in cultures of many cell types make contact inhibition the main subject of the rest of this paper. Contact inhibition is, however, closely related to the phenomena of associative movement; both involve mutual contact of cells, and they may indeed have similar mechanisms; so I should like to add some discussion of associative movement. I shall not be concerned with the recently developing interest in the possibility of a contact inhibition of mitosis.

THE PHENOMENA OF CONTACT INHIBITION

Contact inhibition of movement is a behavioral reaction that is easily seen in cultures of many vertebrate cell types made with a plane surface as substrate (7). It may be defined as an inhibition of the locomotion of a cell in a direction that would take it across the surface of another cell, the inhibition occurring when contact is made between the two cells. Contact here means what appears as such in the light microscope; what sort of a gap there is at the electron microscope level at the time of inhibition has not been determined. The total reaction often looks quite complicated. A fibroblast moving on a plane surface is always led by a ruffled membrane, of uncertain function in locomotion, but certainly strongly correlated with it. A cell that is to be inhibited will therefore make its contact by means of this membrane; some minutes after visible contact the ruffling activity of the membrane dies down and is often substantially paralyzed. The place of contact quickly becomes an adhesion, which it takes some force to break (8) and which may persist for many hours. But the cell itself can break the adhesion. The inhibition of locomotion is a local one

and does not prevent the cell setting up a new direction of locomotion, led by a new ruffled membrane, and the force thus exerted can be enough to free the cell from its adhesion to its partner. The appearance of a new ruffled membrane may be regarded as a somewhat delayed part of the total reaction. The previously leading part of the cell, which has made the adhesion, often seems to develop a tension, which I shall mention again in connection with clumping. This tension may draw the contracting cells closer together if the mutual adhesion is sufficiently high or may at other times draw them apart, presumably when adhesion to the substrate is higher [Paul Weiss' (9) contact retraction]. All these complex phenomena do not invariably accompany contact inhibition between fibroblasts; it is not known how frequently they occur.

It has still to be determined how much, if at all, the mutual alignment of elongated fibroblasts, often seen during the growth of colonies from one or a few cells, depends on contact inhibition, as virologists so commonly assume.

Variations in Incidence of Contact Inhibition

Contact inhibition does not always follow a collision between cells. Sometimes a fibroblast will proceed across the exposed surface of another fibroblast, as if it was part of the substrate. The whole reaction then seems to fail, and we can thus express the amount of contact inhibition as the frequency with which collisions result in inhibition or fail to do so. Unfortunately such collision analysis is extremely laborious, and we do not yet have data of this kind. If we want to compare the amount of contact inhibition in different conditions or between different cell types, we have to rely at present on indirect methods of considerably less reliability. We have used as an index the number of overlaps of cell-on-cell (actually of nucleus-on-nucleus) existing at a given moment in a short-term culture where cells are exposed to mutual collision; the number is standardized against the number to be expected if nuclei of that size and density of population were distributed at random. Because this index depends not only on amount of contact inhibition but on speed of cell movement on top of a cell in relation to speed of movement on the substrate, it needs controlling by other measurements, for instance, of the amount of infiltration there is between the two populations, or by filming. It does not appear that relative speed of movement on cell and substrate is an important variable, but nevertheless small differences in the quantity of overlapping need to be viewed with some skepticism. I should add that multilayering only reflects failure of contact inhibition if it is produced by movement of cell-on-cell; multilayering, built up by associative movement, by mitosis, or by locomotion when the cells are isolated from each other through the presence of collagen, needs to be distinguished (10).

After making the necessary reservations, it is still clear enough that the incidence of contact inhibitions varies considerably. Partly it varies with conditions of culture, which I will discuss shortly, and partly it varies with cell type when cells are in the same conditions. If one compares the overlapping in populations of different kinds of cells, each population consisting of like cells, one finds a range from a level that represents nearly complete contact inhibition (as in adult guinea-pig subcutaneous fibroblasts) to a level which indicates that contact inhibition only occurs in about 50% of collisions (as in mouse Sarcoma MCIM). We have not yet found a cell type which in its homologous contacts shows a complete disregard of fellow cells, *i.e.*, zero contact inhibition. In mixed cultures though, we can find the full range. Barski and Belehradek (11) found for instance that various sarcoma cells will not move at all over chick heart endothelial cells, and we have confirmed this for mouse Sarcoma 180. Yet this same Sarcoma 180 (or perhaps a selected subpopulation of it) shows zero contact inhibition to chick heart fibroblasts and to mouse muscle fibroblasts.

One of the main interests in making quantitative the degree of contact inhibition, homologous (to like cells) and heterologous (to unlike cells), of a number of different cell types is to obtain evidence on the specificity of the interaction. In this context a lack of specificity would mean that all cell types can be arranged on a single quantitative scale of some property, and that the amount of contact inhibition between any two cell types can be predicted from their values on this scale. Insofar as more than one scale has to be used to accommodate all cell types, there being a lack of any regular predictability between scales, specificity is asserting itself. Unfortunately, we lack any data to match those which Steinberg (12) obtained for a different form of behavior which I shall discuss under associative movement. Steinberg found nonspecificity, in the sense mentioned above, for the sorting out of tissues from mixed reagggregates. He could put a series of tissues at least in a rank-order scale, from which could be predicted the relative positions the mixed up cells of two tissues would take after sorting out, on the outside or on the inside of the reaggregate. Since our data for contact inhibition are based on overlaps and are therefore somewhat unsatisfactory, I will confine myself to saying that they point to the existence of a considerable amount of specificity. Only collision analysis will settle this question.

Nevertheless, in spite of the likelihood of specificities in the contact inhibitory interaction between any two cell types, some broad generalizations can be discerned. The most important is that cell types which *in vivo* are able to infiltrate into heterologous cell populations show a relatively small degree of contact inhibition to similar heterologous cells *in vitro* (13). This seems to hold for those sarcoma cells and white blood cells (3) that have been tested. It is also thought by some that sarcoma cells tend to show a relatively slight contact inhibition by homologous sarcoma cells. On the other hand, within the general class of fibroblasts there seems to be

a well-developed contact inhibition, both to homologous and to heterologous fibroblasts. These generalizations particularly concern the relationship of *in vitro* to *in vivo* behavior, and I shall return to them.

The assertion of the existence of intrinsic differences between cell types in their contact inhibitory behavior should not of course be taken to mean that in given conditions a given cell type is the same throughout its history. It does seem indeed that cells in mitosis lose much of their contact inhibitory power; and we might expect important and longer lasting changes during ontogeny, though these have not been investigated. The only long-term change in the intrinsic property yet followed *in vitro* is that produced by oncogenic agents (14-17).

Environmental Influences on Contact Inhibition

There is one very great difficulty in studying the intrinsic resemblances and differences of cells in their contact inhibition, and that is their great susceptibility to environmental influences in manifestation of the property. The caution that is required is illustrated by the difference between chick embryo heart and neonatal mouse muscle (18). In carefully standardized cultures of each of these cell types, the chick cells show considerably more homologous contact inhibition than the mouse. But in such cultures, conditions are not really standardized in one respect. Chick-chick contacts take place in an atmosphere created by other chick cells, and mouse-mouse contacts in one created by other mouse cells. When this environmental difference is removed, by mixing chick and mouse cells in a single culture, the homologous contact inhibition of the two types is indistinguishable. (It should be added that in mixed cultures of other cell types, the different cells have become more different rather than less.) Evidently neighboring cells create conditions affecting the outcome of individual collisions, and this is borne out by the fact that within a homogeneous population the intensity of contact inhibition is negatively related to the density of population (19). It is already clear that there are many more constituents of tissue culture media, and other substances, which influence the amount of contact inhibition.

Another class of environmental condition that affects contact inhibition is the nature of the tissue culture substrate on which the cells are moving. In media containing serum, which inevitably coats the substrate to some degree, we have found on the whole little effect on the amount of contact inhibition of a variety of standard substrates, such as glass, collagen, fibrin, and different plastics. It is, however, clear that there are a few surfaces to which fibroblasts and probably other cells, also in serum, adhere very poorly, and that these surfaces diminish contact inhibition. Associative movement very easily occurs on such surfaces: That is to say, the mutual adhesion of the cells overcomes the adhesion to the substrate and the cells

draw together into groups with much overlapping. Such overlapping has to be distinguished from that produced by failure of contact inhibition. The latter should produce a random, not a clumped, distribution of cells, and it is desirable also to check by means of filming whether or not the cells are moving across each other, led by ruffled membranes. Carter (20) used an ingenious and controllable nonadhesive substrate consisting of cellulose acetate shadowed to varying degrees with palladium. With L cells he found on the less adhesive parts of the substrate, a great deal of overlapping, most of which seems to have been due to clumping. But he noted that in a narrow range of substrate adhesiveness the cells moved apparently normally, but with much overlapping, which must have represented some breakdown of contact inhibition. We have found a similar situation on agar or agarose, also a relatively poorly adhesive substrate. It seems to be possible to vary the ease with which fibroblasts attach to and move on an agar gel by varying the concentration (by drying a film of agar before adding medium). In conditions of adhesiveness near to those that produce clumping, primary chick heart fibroblasts will move on the agar surface and show a substantial loss of contact inhibition. The amount of overlapping may rise from about 10 to about 30% (sometimes as high as 50%) of that expected if the cells were distributed at random. They do not, however, become fully randomized, which would represent a total loss of contact inhibition. The partial loss seems to occur immediately when the cells find themselves on agar, and the usual contact inhibition is regained as soon as they move from the agar to a standard substrate.

The Mechanism of Contact Inhibition

Exploration of the mechanism of contact inhibition, as of so much else in cell behavior, is severely handicapped by lack of knowledge of the way cells move. Judging from our present information, it seems that perhaps one should not look for one single straightforward mechanism. That the phenomenon is probably complex in fibroblasts is hinted by the apparent specificities in the intensity of inhibition between cells of different types. Different cell types may furthermore, perhaps, put different weight on different mechanisms. The description I gave at the beginning of this paper of the act of inhibition as seen in a fibroblast applies to a considerable extent to an epithelial cell. But the adhesive, paralytic, and contractile features of the process in these cells are by no means obvious in the homologous collisions between some sarcoma cells which show a good deal of mutual interference with overlapping; the same applies more strongly to leukocytes of various kinds, which have a pronounced tendency to form monolayers (3).

In fibroblasts there are good grounds for saying that contact inhibition, as its name implies, really does depend on some special relation of the contacting surfaces, stemming therefore from the short-range affin-

ities and repulsions of surface constituents. Two other possibilities can be regarded as unlikely. The first is that the locomotory machinery will not carry the cell over an obstacle, such as another cell, but can only operate in a direction parallel to the substrate. But on the contrary, fibroblasts will surmount considerable obstacles of glass or fibrin without inhibition, though some obstacles may divert them, probably by contact guidance (21). The second hypothesis that seems at present unlikely is that contact inhibition works by means of something that diffuses between the cells. No sign of any incipient inhibition is detectable until the cells touch. And presumably this mechanism would operate as a block to free diffusion of a substance produced by both cells, producing a "boundary chemotaxis." (A hypothesis involving *different* substances produced by the two contacting fibroblasts is hardly tenable, particularly since a collision of two ruffled membranes, *i.e.*, physiologically similar parts of similar cells, will produce an inhibition.) If this is so, inhibition should occur at inanimate obstacles, which it does not seem to do. To these general arguments, one can add a special one apropos of a recent suggestion (2) that acid might be the agent involved: It is the notorious acid-producers, the tumor cells, that fail to inhibit fibroblasts. But a diffusive mechanism might be concerned in the monolayering of white blood cells (3) because, as I have mentioned, there are clear indications that these cells have in their repertoire of behavior a mutually repulsive chemotaxis.

We have then to seek a contact-dependent explanation, or complex of explanations, at least for fibroblasts. Unfortunately, it is not clear yet what the nature of this explanation needs to be. It may be worth setting out the problems by following through one line of thought that has recently been investigated.

All movement on a smooth solid substrate must involve a cycle in which adhesions are made to the substrate toward the front of the cell, and the same adhesions are later detached when the body of the cell has moved forward in relation to them. It may be supposed that the detachment of the old adhesions requires the presence of the new adhesions, in the sense that the old are pulled off the substrate by means of the firm attachment of the new. This, however, cannot be the only mechanism involved in disposing of the old adhesions. To some extent the firmness with which they are attached must decay independently. Otherwise, on a homogeneous substrate, there is no reason why it should be the old rather than the new adhesions that are pulled off.

Consider now what will happen when a cell, migrating on an otherwise homogeneous substrate, runs into a patch of substrate that is less adhesive. If the decrement in adhesiveness is too great, it will be unable to proceed on to the patch, because it will be unable to detach its old adhesions. If the decrement is small, however, the decay of the old adhesions may permit the new ones on the nonadhesive patch to win in their competition with the old.

Fibroblasts seem to behave, as far as they have been tested, exactly as required by this hypothetical picture. They will not move from a strongly adhesive substrate onto a much less adhesive one. Carter (20) has shown that L cells will not move from glass to cellulose acetate. We have found that they will not move from collagen to dilute agar or agarose. On the other hand, we find that they will move from collagen to an agar or agarose patch that has been highly concentrated through preliminary drying. We know that such a patch is relatively nonadhesive because of the ease with which the fibroblasts that get onto it come loose and retract back onto the collagen, and by the great difficulty there is in getting explants to attach to such a surface. These results clearly make the point that fibroblasts achieve the necessary de-adhesion during their locomotion both by competition with the new adhesions and by independent decay. If they did it in the former way alone, they would not be able to move on a homogeneous substrate, let alone transfer to a less adhesive one. If they did it in the latter way alone, they would be indifferent to change of substrate.

All that we have to do to provide on this basis a theory of contact inhibition is to suppose that the fibroblast surface onto which other fibroblasts are unable to move is very nonadhesive (22). Actually, of course, fibroblasts stick rather firmly together; but this is by their lateral edges, when they are on a plane substrate, and the relevant nonadhesive part would be the main expanse of their surface that is exposed to the liquid medium. It follows from this theory that, if we decrease the adhesiveness of the substrate, we should reach a point when the decay of the adhesion to the substrate is sufficient to allow the cell to move onto another fibroblast. In fact, this seems to happen, as already mentioned: On relatively poorly adhesive surfaces there is a certain degree of inadequacy of adhesion at which contact inhibition becomes reduced.

It might be supposed that sarcoma cells have an exaggeratedly independent decay of their locomotory adhesions to substrate, so that they are relatively indifferent to variations in adhesiveness of substrate, and thus move freely over fibroblasts. We have, however, found that they move *less* easily from collagen onto concentrated agar than do fibroblasts. We must at present conclude that they have a different scale of adhesiveness from fibroblasts.

The basic idea that differential adhesion to cell and substrate is the cause of contact inhibition seems at present satisfactory, but it is not wholly satisfactory. The common accompaniments of contact inhibition, the mutual adhesion, paralysis of ruffling, formation of a new ruffled membrane, and contraction are not accounted for by differential adhesion. Neither do they occur when a fibroblast is stopped by running into a patch of nonadhesive agar; it may stay at the border for hours, ruffling away.

The common accompaniments may turn out not to be causally related to the inhibition of movement. But it seems to me that at present we must be prepared to envisage a second contact-dependent mechanism operating in parallel with differential adhesion and capable of producing some contact inhibition when differential adhesion fails. This mechanism, it may be suggested, is an effect of adhering strongly to the contacting cell. The adhesive interaction perhaps produces a cell membrane change [recent observations (23, 24) on electrical conductivity show that contacts may be the site of functional cell membrane specializations] and this in some way signals a cessation of the locomotory machinery, checks the ruffling, and so on. However, the proposal that there are two independent mechanisms of contact inhibition in fibroblasts has itself unsatisfactory features—notably the requirement that the fully developed malignant fibroblast must be assumed to lose both.

The numerous references to degrees of adhesiveness that I have made need, of course, to be translated into molecular terms. As yet, it does not seem that knowledge of the makeup of cell membranes and the forces likely to act between two such membranes or between membrane and substrate throws any clear light on contact inhibition. It seemed at one time that a reduced contact inhibition, such as has been found in some malignant cells, might be a reflection of a nonspecifically low adhesiveness of the cell surface (7); and this in turn might be a reflection of a high negative charge density on the cell surface (25). It is not, however, clear that, as compared with nonmalignant cells, the adhesiveness of malignant cells is nonspecifically reduced (26, 27); and the increased surface charge observed may be a concomitant of a high rate of proliferation rather than of malignancy (28–30). The hypothesis relating adhesiveness to contact inhibition via surface charge seems therefore to have been too simple.

ASSOCIATIVE MOVEMENT

We have so far considered in contact inhibition a dominant mechanism of control of the orientation of solid substrate locomotion *in vitro*. Contact guidance and occasionally chemotaxis are other control mechanisms of this form of locomotion. Associative movement, which I now want to consider, is really to be regarded not merely as a control mechanism for solid substrate locomotion, but as a different form of locomotion. It is a form of behavior such that when cells make contact they form adhesions by means of which the cells draw closer together and maintain themselves in close contact. Just how different the machinery of this movement is from that of solid-substrate locomotion will only appear when something is known about the physiology of movement. But it seems distinct

in its outcome, and at least the two forms of movement are at the extremes of a continuum, in that associative movement is a movement that draws the cell *towards* what it is adhering to, and consequently does not move it far; while solid-substrate locomotion, by its cyclic nature, shears the cell *across* whatever substrate it is adhering to, and can go on indefinitely. The displacement produced by associative movement is obviously not orientated by contact inhibition nor by contact guidance in the classic sense; it is orientated by the position of the relevant adhesion.

Associative movement will obviously influence the direction taken by solid-substrate locomotion. Indeed, the two forms of displacement will in ordinary tissue cultures frequently be in opposition, since solid-substrate locomotion will often tend to carry cells apart, by random (diffusive) movement, and more strongly under the influence of contact inhibition. Associative movement manifests itself most clearly when solid-substrate locomotion fails, which may happen either because the substrate is substantially nonadhesive or because the cells are in suspension. But it doubtless shows its influence, in equilibrium with solid-substrate locomotion, in the varying degrees of association of the cells of ordinary tissue cultures, from the closely packed epithelium, through the fibroblast meshwork, to the discretely dispersed wandering cells. This view of the architecture of cell colonies is of course long established (31-33). I have already noted that in fibroblasts incipient associative movement, in the sense of a slight drawing together of the cells, may appear as soon as contact inhibition between colliding cells has occurred.

Many examples of the effects of associative movement could be given. The really interesting cases from the morphogenetic point of view that occur *in vitro* concern mixed populations of cells. It is very well known through the work of Moscona (34) that, when mixtures of dissociated cells from different embryonic tissues are allowed to clump, the cells within the resulting aggregates sort themselves out histotypically into distinct regions. I have already referred to Steinberg's analysis (12), in which he showed that the tissues could be placed on a single ordinal scale of some quantity whose relative values in any two tissues determined which should become external in the aggregate. His argument, that the quantity involved represents adhesiveness and that the whole process of sorting out can be explained by the tendency of moving cells to maximize their adhesions, seems to me convincing. As Steinberg has pointed out, this explanation of sorting out by relative adhesiveness is closely analogous to the explanation, already considered, of at least an important component of contact inhibition by relative adhesiveness. Now it may be that, even in sorting out, the movement involved is solid-substrate locomotion. My feeling, however, is that the rather small cell displacements involved in sorting out (35) will prove to be of the short-range associative movement type.

RELATIONSHIP OF BEHAVIOR MECHANISMS *IN VIVO* TO THOSE *IN VITRO*

I referred at the beginning to the orientated movements and the resulting patterns of cell distribution that occur in tissue cultures. For fibroblasts they include the predominantly radial emigration from an explant, monolayering, the circular form, and the usually sharply marked periphery of many colonies. Other cell types show different patterns. Most of these seem to me to be analyzable at a behavioristic level, and in many cases very fully analyzable, as the necessary consequences of well-established cellular reactions. There are some fibroblast patterns that raise questions, such as their parallel alignment and in older cultures the occasional orthogonal arrangement of superimposed layers; but on the whole we can feel that for fibroblasts at least the repertoire of behavioral reactions that comes into play in cultures is becoming pretty well sketched in. It is clear though that we are a long way from understanding the physiology of any of these behavior mechanisms.

There is no such confidence that the behavior mechanisms manifested in culture also account for the orientated cell movements in the whole organism. It is not indeed certain that conditions *in vivo* are sufficiently like those *in vitro* for the behavior mechanisms we have analyzed to work at all *in vivo*. We have to be particularly cautious in the case of contact inhibition because of the evidence from many sides that monolayer culture conditions rapidly change cells. Among other things, they apparently change the properties of the cell surfaces, as indicated by the severe decline, after a few days as a monolayer, in the ability of cells from young embryos to aggregate when resuspended (36, 37). Such a change in surface properties may be correlated with a change in contact-dependent behavior, and it cannot be ruled out that the tissue culture environment, particularly perhaps the plane surface of the substrate, may induce or exaggerate contact inhibitory behavior. A similar diminution of ability to reaggregate occurs, though more slowly, during the normal aging of embryonic tissues (36, 38); and this, together with direct observation of the apparent intensity of contact inhibition of cells as they emerge from an explant, suggest that tissues from an older embryo or postembryonic stage may not be so subject to this hypothetical enhancement of contact inhibition by culture conditions. It must be admitted that, if contact inhibition is deficient in young embryonic tissues *in vivo*, it is puzzling what keeps these highly mobile cells in place in the embryo. That they do remain in place when they are part of a tissue is strongly indicated by tritiated thymidine marking of parts of organ cultures (39).

Whatever tissue culture does in the way of immediate induction of contact inhibitory behavior, it seems that in the long run it tends to select against such behavior, as Todaro and Green (40, 41) so well demonstrated with their 3T3 line of fibroblasts.

When two directionally migrating populations of fibroblasts are made to collide in culture, the movement of the cells is brought virtually to a standstill. The mechanism involved is evidently contact inhibition, which blocks the movement of each cell in all directions (42). A similar cessation of movement on contact occurs with opposed epithelial sheets *in vitro*, and it can be readily observed to occur on the surface of a whole organism. It was indeed recorded *in vivo* long before the theory of contact inhibition was introduced for fibroblasts (43), and it has been carefully analyzed by Lash (44). It seems probable that here we have a direct observation of the operation of contact inhibition *in vivo*.

A second line of evidence suggesting that contact inhibition is significant in the whole organism comes from the cells that are deficient in it. There seems to be an association between an ability to infiltrate connective tissues *in vivo* and a low degree of contact inhibition against fibroblasts in culture, in that both leukocytes and those sarcoma cells that have been tested combine the two properties. This suggests that contact inhibition may play a part in preventing the invasion, by proliferating normal cells, of neighboring tissues (7). It is desirable to establish the degree of correlation between contact inhibition by collision with normal cells on the one hand and invasion of similar normal cells *in vivo* on the other, before the association can be acceptable, and this awaits a method of assessing invasion quantitatively. I should add that the association that I and my colleagues have proposed is not tested by comparing the *homologous* contact inhibition, *i.e.*, between like cells, with the transplantability of those cells to an organism (45, 46)—particularly when the degree of homologous contact inhibition is estimated by a method (assessment by eye of multilayering and crisscrossing of cells) that may or may not be an index of contact inhibition of movement as we have defined it.

There seems little doubt that the kind of movement I have called associative occurs in the whole organism. Perhaps the best studied examples are found in the gastrulation of the echinoid embryo, described by Gustafson and Wolpert (47). The selective kind of associative movement, that is the histotypic sorting out of reaggregated cells which was discovered *in vitro*, is a different matter. On the face of it, it seems entirely possible that this is an artifact produced by the disaggregation procedure and by the unaccustomed neighbors that the cells find themselves alongside. Steinberg (12) has, however, brought forward cogent evidence, in my opinion, that the cell properties involved in sorting out can be active in organized embryonic tissue. He showed that precisely the same external-internal arrangements are taken up when tissues sort out after disaggregation as when fragments are simply placed in apposition and allowed to fuse.

The direct evidence that the *in vitro* behavior mechanisms I have discussed occur also *in vivo* is evidently somewhat tenuous at present, but at

least it is compatible with the idea. Assuming that they do, I will conclude by considering in what morphogenetic episodes of the organism these mechanisms are from their nature likely to take part.

Contact inhibition, as I have already discussed, may well have a role in the restriction of long-range cell movement in organized tissue, that is to say in the maintenance of organization. Its involvement in morphogenetic movement seems to me likely to derive from its concomitant effectiveness as a filler of space. It directs cell movement into cell-free space, and when that space is filled it stops the movement. In this self-regulatory process it may be aided by contact inhibition of mitosis (48). As a filler of space, it may be suspected that its main role is in processes of repair, which are often highly analogous to the development of a tissue culture. Associative movement I would regard as a major agent in making the fine adjustments of cell position that give tissues and organs their intimate cellular structure. It can be much refined in its outcome if it occurs between cells with unlike, and changing, surfaces on different parts of their anatomy. It may obviously play such a role in the formative phase of the embryo. The decline with age in the ability to achieve sorting out in experimental conditions (36, 38) suggests that in later stages associative movement may be less important; but the problems the experimenter sets to the cells in sorting out from reaggregates are extremely severe, and the continual fine adjustments required during postembryonic growth may still be within the competence of the mechanism.

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DISCUSSION

Dr. Lash: Dr. Abercrombie has shown beautifully how one can dissect various aspects of cell movement *in vitro*, namely, contact guidance, contact inhibition, and associative movements. These events are usually studied as isolated events in tissue culture, but I think they can also be analyzed, or at least looked at, *in vivo*. Figure 1 shows an *in vivo* example in amphibians where the phenomena of contact inhibition, contact guidance, and associative movement all occur in the same tissue. Figure 1 is a cross section of an amphibian epidermis showing various cell types. The cell types include large gland cells, small epidermal cells interspersed between the larger cells, flattened cells at the surface, and various types of pigment cells. If the epidermis is injured, this epithelium will move as a sheet to cover the wound, as illustrated in figure 2. It is this process of wound closure that I think exhibits three of the phenomena Dr. Abercrombie mentioned.

The first is contact inhibition. When this epithelium moves across the wound surface and meets the advancing sheet from the other side, both the epithelium and the sheet stop. This is an example of *in vivo* contact inhibition.

In contact guidance, it was shown that the epithelium needs a substrate upon which to move. Only if the epithelium is in contact with an appropriate substratum will normal closure take place.

As for associative movement, this is a term I wasn't aware of when this work was done. Only after reading Dr. Abercrombie's manuscript did I look back at it. There are examples of what one might call associative movement during wound healing. In many instances during closure, the smaller epidermal cells migrate together and form small clumps (fig. 3). These clumps move as a unit in the epithelial sheet. Whether this associative movement is responsible for the epithelial movement I don't know, but I am sure this movement of the smaller epidermal cells is associated with that of the epidermis.

Another point Dr. Abercrombie mentioned is the differential adhesion of cells and its relation to their movement. The amphibian epidermis is a heterogeneous tissue. If differential adhesion has anything to do with tissue movements, maybe these cells have differential adhesive properties.

If the epidermis is disaggregated and put back together, the cells should adhere to one another in a particular pattern if they have differential adhesion. Figure 4 shows the reaggregation of the epidermal cells. The cells come together as very tight clumps as if they are doing all they can to get in tight contact with one another. Figure 5 shows that these clumps are homogeneous in cell type. Each clump consists of only one cell type, *i.e.*, all white pigment cells, black pigment cells, epidermal cells, etc. These cells all show differential adhesion in that they only adhere to cells of their own type.

If one looks hard enough at *in vivo* situations, it is therefore possible to find examples of Dr. Abercrombie's phenomena. These phenomena will not occur isolated as studied in tissue culture, but will usually be cooperative events associated with tissue movements during development or wound healing.

Dr. Abercrombie: I am delighted that Dr. Lash brought up this admirable earlier work on the behavior of epithelial wound healing for two reasons:

- 1) This introduces epithelium, while I was talking entirely about fibroblasts, and indicates that phenomena similar to those we find with fibroblasts do seem to occur with epithelium. We have found the same kind of behavior *in vitro*. If you make two epithelial sheets collide, they stop each other.

- 2) Dr. Lash's work has the tremendous advantage of demonstrating these phenomena *in vivo*. It is almost unique in this and shows, therefore, that the kind of analysis we do *in vitro* can be applied to the whole organism, at any rate in these respects.

Dr. Lash also mentioned an extremely interesting and strange phenomenon, an aspect of associative movement, when he showed that dissociated epithelium from a healed wound aggregated from the very beginning according to tissue type so that each aggregate was of a different kind of tissue. I think this is unique in vertebrates, but no doubt Dr. Moscona will correct me if this is wrong.

What it does seem to me to show is how one can affect the behavior of cells in these contact relationships by their treatment. Because these cells have been dissociated, they show this remarkable aggregative behavior *in vitro* which must, I think, indicate that these cells are very much more adhesive to other cells of their own kind than they are to heterologous cells.

But *in vivo*, surely, in this epithelium, this extreme differentiation of adhesiveness cannot be present. Therefore, the effect has been produced by the treatment. This is a warning of the kind of effects that one may expect to get by particular treatments on these kinds of contact behavior.

Dr. Moscona: I have listened with great interest and fascination to these two masterly exposés of topics that are obviously closely linked by an intercellular substance. I am rather delighted with the general point of view expressed by both

speakers because it seems to me they both are essentially in agreement with the kind of concept that advocates the role of the cell surface in developmental phenomena—the kind of concept we have believed in all along. This concept stresses the possibility that the cell surface is endowed with considerable dynamic properties that change in response to extrinsic conditions as well as in response to regulatory mechanisms intrinsic to the cell.

We have long ago espoused as a possible working hypothesis the idea that the cell surface, in addition to the double-layered physiological membrane, is also endowed with a molecular array of macromolecular products which, at the cell surface, between cells, and in an extracellular location, endow the cells with properties usually associated with cell activity, with contact, with preferential adhesiveness, and related phenomena.

The time has come to consider these possibilities, particularly in view of what was and what was not said here but placed in the manuscripts. We must consider these possibilities in more profound terms and ask ourselves whether these products at the cell surface by virtue of their molecular properties might not be mediating the sort of phenomena Dr. Abercrombie was talking about when he referred to differential adhesiveness and Dr. Grobstein was talking about when he referred to interactions, adhesions, and selective adhesions between tissue components and cells.

I also am delighted that Dr. Abercrombie is addressing himself more and more to the possible correlation between the contact inhibitory phenomenon *in vitro* and aggregation of phenomena *in vitro* and *in vivo*.

I am not particularly happy with the term "associative movement" because it connotes the possibility that there are all kinds of movements. I would rather see the term "aggregation" retained and "movement of cell aggregates," "movement of cell clusters," or "movement of cell groups" retained rather than involve ourselves in terminology that might be misleading in that we might look for phenomena which would justify the term.

Concerning contact inhibition, with Dr. Abercrombie's permission and indulgence. I would like to be a sort of Devil's Advocate and ask a number of questions which I am sure he can answer.

Is it conceivable just by stretching our imagination that contact inhibition phenomena are essentially symptomatic of adaptation of cells to existence in the monolayer? For instance, is it only when cells undergo the kind of changes that we know many cells undergo rather rapidly in monolayer that they begin to exhibit the sort of properties we usually lump under the term "contact inhibition"?

As these cells change in monolayer, do they acquire presumably surface properties reflecting these changes? What they may acquire is a surface material, an extracellular material, which is different in response to the conditions and in response to the genomic adaptation of the cell. These changes might then be reflected by what we see as a measure or can measure, or can less than measure as contact inhibition.

Under certain conditions, which promote not adaptation to monolayer but persistence of phenotypic traits in monolayer, is it then possible that such phenomena, which we term "contact inhibition," would not occur and that differentiating cells in monolayer would show less contact inhibition than differentiating cells in monolayer?

Is it then possible, for instance, that differentiation and acquisition of the property of contact inhibition are contrary to the tendency of cells to aggregate and form histogenetic association?

Then might one modify or manipulate the culture environment so as to direct cells toward acquiring a contact inhibitory behavior rather than retaining an associative behavior that is perhaps more normal?

Dr. Barski has examined these kinds of possible changes in the phenomic expression of the cell. He studied the metabolic profile of the cell as it changes under various

culture conditions and neoplastic cells and got some exceedingly interesting results. He tried to correlate contact inhibition of the cells in a monolayer system with their aggregatability when challenged in the rotational flask testing system.

I am sorry that Dr. Abercrombie did not expand on the phenomenon of mitotic inhibition in terms of contact control. The theory of the relationship between cell density and mitotic activity was really started by Willmer, I think, in the early 1930's. It has been shown that this relationship exists. But today, particularly, due to the work of a group of Harry Eagle and a great many others, it is possible to examine this in terms of molecular events. It is beginning to look as if contact affects the metabolic profile of the cell and is, in turn, itself affected by changes in the metabolic property of the cell.

In other words, there is a close relationship between contact phenomena and changes in the function phenomena, possible genomic, of the cell. We have to face this possibility now. The phenomenon of contact-controlled mitotic processes affords an opportunity for such investigations at a perhaps sophisticated level.

Finally, even contact inhibition, in the terms that Dr. Abercrombie was talking about, might lend itself to scrutiny at the molecular level. For instance, we would like to know if contact inhibition in your terms would work across the filter membrane of the sort that Dr. Grobstein was talking about. We would like to know whether contact inhibitory phenomena can be effected by molecular interference that might change the properties of the cell surface.

Can we reconcile at the moment the fact that tumor cells which do not contact-inhibit each other nevertheless respond to contact inhibition by normal cells and the fact that the phenomenon of contact deinhibition of tumor cells is not general? At the same time, contact inhibition in nontumor cells is also not general.

What I have tried to do here, I hope, is to raise a number of questions I am sure all of you have had in mind. These questions are extremely pressing and only serve to indicate the tremendous progress that the Abercrombie and Grobstein groups have made, and the tremendous significance of the issues which they have placed before us to study.

Dr. Abercrombie: Dr. Moscona has raised many points, to all of which I would like to reply at length, but I shall restrain myself as far as I can. First of all, I absolutely agree with Dr. Moscona with regard to the question of macromolecular products at the cell surface. I feel that the contact inhibition work requires such rather specific substances. I don't think that contact inhibition can be analyzed simply in terms of some nonspecific kind of adhesiveness between cells.

We find specificities, in fact, in the relations between cells. This seems to require particular conformations on the surfaces which will react. I am sure that the same is true of the sorting-out phenomena, the phenomena which I have included under associative behavior as they manifest themselves in aggregates, at any rate, when you reach a certain degree of complexity. I think that Steinberg's results cannot really easily be interpreted in any other way than by a single scale of adhesiveness as he does. But I feel that this applies to newly dissociated cells, and one may get much more complicated situations when cells have recovered from the dissociation procedure.

Then, one may get differences in adhesiveness which depend, I imagine, on macromolecular products at the cell surface.

I am not satisfied with the term "associative movement," but I intended to include a larger number of phenomena rather wider than is at present included under the term "aggregation." This was the only reason for not using "aggregation." I mean, for example, the kind of behavior that the early tissue culturists called attention to and interpreted in terms of mutual behavior, *i.e.*, the difference in culture between epithelium, fibroblasts, and wandering cells in their degree of association. But I am not at all committed to the term.

The really important point is the adaptation that may occur in the tissue culture situation which may induce contact inhibitory behavior in cells. I think that this could easily come about through contact with a plain surface.

It could well happen with the younger embryonic cells. These are the ones that Dr. Moscona himself has shown changed rather quickly in their surface properties when in monolayer culture. They lose rather quickly their ability to aggregate. This evidently indicates a surface change that might well be the appearance of the kind of surface which produces contact inhibitory behavior. This coincides with my own feelings from simply looking at cultures of young embryonic cells. It probably applies distinctly less, if at all, to cultures of much later embryonic tissues and of adult tissues that do not aggregate well in the kind of system Dr. Moscona has used and seem to show contact inhibitory behavior from the first moment they are put into culture.

This is an extremely important point. What it might mean is that in the early embryonic stage contact inhibition has no particular role to play, while, as I suggested, its main role would be in the later stage of the developed organism in wound healing and regeneration.

I have not commented on mitotic inhibition because I know much less about it than some other people who have studied it extensively. My own hesitations about it are simply that I am not sure whether it has been shown to be a contact effect. But there is an effect of proximity. This is clear from Dr. Willmer's work. Albert Fischer also had the same ideas. I am not certain that proximity, working not through contact, but through the production of nondiffusible substances—maybe these macromolecules at the surface which Dr. Moscona has referred to—could not still account for the experimental results.

This, I know will be solved very quickly. And I ardently want it to turn out to be a matter of contact, not of anything else.

One final point is that, if contact inhibition could occur between cells separated by membranes, this would nullify the whole idea of *contact* inhibition. I have never thought that this was likely because one never sees any effect of the approach of cell populations toward each other until they actually make contact as one can see it in the visible microscope. There is no apparent diminution of movement at a distance which would suggest a diffusive substance.

Dr. Zwilling: My comments about Dr. Abercrombie's paper and his latest remarks will really be a return to a very unsophisticated level and will deal to a great extent with something that most of us know.

I would like first to emphasize what we have all been becoming more and more aware of recently—namely, the importance of cell surfaces, especially from my interests in cells during early development. We all hope that something equivalent to the phenomenon that Dr. Abercrombie has been dealing with is behind some of the very striking movements occurring in the early stages of cell development.

I wonder whether one aspect of the problem resides in the fact that many of these surface properties may be transitory in early embryonic tissues. I would like to remind everybody about the striking dissociations and new reassociations that occur during early development of the cell. Probably the most common example of this is the history of neural crest cells, which first start out as part of a continual sheath of tissue. The cells then leave the sheath more or less individually, migrate relatively long distances, and then reassociate in various ways.

The phenomenon of cell sorting, which is usually associated with histotypic development and histogenetic capabilities, has recently been shown in my laboratory to be associated also with morphogenetic properties and to be independent of the histogenetic properties.

Briefly, when we randomized cells with equivalent histogenetic capabilities from somites and limb bud mesoderm, both these tissues were capable of making cartilage,

muscle, and connective tissues. These sorted on the basis of the rudiment of origin whether they came from somites or limb bud mesoderm and not on the basis of whether they could make cartilage.

Again, this phenomenon appears to be transitory because, when we isolated chondrocytes from vertebral cartilage and intermixed them with chondrocytes from long bone cartilage, no sorting occurred.

Dr. Abercrombie: Dr. Zwilling has raised a cardinal point for developmental biologists; *i.e.*, if these various contact systems are to be made to produce the morphogenesis of embryos, then they have to undergo temporary changes, transitory changes as he suggested. This is difficult to investigate but, for the embryologist, is extremely important to follow up.

I should add one minor point: Not only must the cells change in time, but they must also have different properties on different parts of their surfaces, these, too, presumably changing with time.

I am sure that one will find many complexities in the surface properties of cells. Of course, Dr. Grobstein is really investigating the way that these kinds of surface properties concerning morphogenetic movements are shifted during development by tissue interactions.

Dr. Zwilling: May I make one point with respect to your remark and in addition to what I said? Very frequently, the time interval required for an experiment represents the interval during which a transition may take place with respect to these properties.

Dame Honor Fell: Since the panelists have no further remarks to make at this stage, I am going to take an unfair advantage of my position as chairman to make the first comments from the floor. I want to offer a suggestion for the future.

There is one aspect of cell physiology, which I mentioned very briefly in an earlier discussion, that I feel ought to be considered in connection with cellular movement and interrelationships. This is the lysosomal system. Observations in our Laboratory have shown that, in skeletal tissues in organ culture, cells normally release a certain amount of lysosomal enzymes into their environment; this release can be very much increased by such agents as excess of vitamin A or complement-sufficient antisera, the greater release being compensated by a greater synthesis of the enzymes. Where cartilage is concerned, hydrolases liberated in this way digest the intercellular material more rapidly than it can be replaced by synthesis, and consequently the tissue disintegrates. The cells, however, remain viable, assume a spindle shape, and become mobile. These changes are reversible when the explants are transferred to normal medium.

The excessive release of lysosomal enzymes and consequent breakdown of matrix produced by vitamin A or antiserum can be largely inhibited by addition of hydrocortisone (as little as 0.1 $\mu\text{g/ml}$) to the medium.

These findings suggest some questions in the present context that perhaps deserve investigation. For example, as a cell differentiates, do the properties of its lysosomal system change, so that the cell either acquires or loses the capacity to break down materials at its surface and, correlated with this, acquires or loses the capacity to migrate? Can one cell type activate or inhibit the lysosomal system of another and so perhaps alter the properties of the plasma membrane? At present we have no information about these points. It is perhaps relevant to this discussion to mention that, in the normal adult, the lysosomal system differs widely, in both its physiological properties and its response to chemical agents, in different organs of the body.

So perhaps we ought to try to find out whether the lysosomes play any part in the interrelationships and movements of cells during embryonic development.

I think you have some ideas about this, Dr. Abercrombie.

Dr. Abercrombie: Not ideas, really. I think this is something which those interested in the role of the cell surface and morphogenetic movements have completely neglected, and it is important that they should take some notice of it.

It just so happens that one of the treatments of fibroblasts which does severely diminish their contact inhibition is treatment with cell-specific antibody. Antibody in the absence of complement is an exceedingly mild treatment producing no effect that we have detected on the cells, except that it greatly diminishes their contact inhibition. It is known that antibody in the absence of complement releases lysosomal enzymes, and this may be the explanation of what we have seen.

Dr. Barski: Certainly, Professor Abercrombie had the very remarkable prescience to attract attention to phenomena that were seen and were rather easily accessible to observation but which remained strangely outside any formulation. He introduced this notion of contact inhibition. How badly it was needed can be measured by the frequency of use of this formula in the literature now. But sometimes it is used and abused. Nevertheless, Professor Abercrombie had the merit to open the way and to interest people in studying the behavior of cells and behavior of cells in relation to their malignancy or appearance of malignancy to such an extent that many virologists are relying on the phenomenon of crisscross and random cross to decide about the malignancy. However, they are forgetting to inoculate the substance into the animal to confirm this statement.

We tried to enlarge, confirm, and formulate, perhaps in a more detailed way, this phenomenon, which is especially visible in cells when they convert to malignancy *in vitro*. We used essentially two systems.

One was to study the mobility of cells in populations of established lines in relation to their malignancy, where the malignancy could be and was systematically defined by inoculation.

Here, we used Dr. Sanford's strains. Essentially, there are two strains—the low-malignancy strain 2555 and the high-malignancy strain 2472 which were used in our hybridization experiments. The difference in their behavior when studied by time-lapse cinemicrography in pure populations was striking. The low-malignant line actually formed monolayers, practically without overlapping, that were epithelial-like. But one must remember that these two lines derive from one clone, and this clone is from subcutaneous connective tissue. So there is no doubt that they are not of epithelial origin. But can we actually say that, when these low-malignant cells come into contact, their leading membranes are paralyzed? No. They continue to move in contact.

Similarly, in another example of a low-malignant line obtained from lung tissue of known histological origin, it is not excluded that this normally was actually a nonmalignant line; it is of epithelial origin. Here, we observed what actually occurred when two cells of the low-malignant line came into contact, and the essential phenomenon is not so much paralysis on contact but a tendency to interconnection.

According to these observations, we believe that the essential phenomenon is a kind of solidarity of the cells. The cells are interdependent by contact. This contact occurs very rapidly. They can dissociate and re-form the contact again. This is a very important point, especially when we study the mutual behavior of malignant and nonmalignant cells. However, highly malignant cells are very similar, whether of the C3H origin of Dr. Stanford's lines or from the C57BL mouse as in our case.

The essential observation is that they are very mobile, not only in the sense of locomotion, *i.e.*, displacement of the cell in the field of observation, but also especially in their processes. These processes overlap, and this overlapping, this interconnection, can be responsible for many phenomena and essentially for the phenomenon of clumping when the cells are suspended. Applying Dr. Moscona's technique of steering of the cells, we were actually very annoyed by this property because the cells came off the glass very easily and formed unwanted clumps.

The second essential group of investigations in our laboratory concerns the interaction of malignant and nonmalignant lines. Professor Abercrombie mentioned that the essential observation was that the issue of the confrontation depends on the nature of the malignant cells as well as on the nature of the normal cells with which they are confronted.

The first observation was that the malignant cells were inhibited by normal endothelium. This is now confirmed by Dr. Abercrombie. We have also seen that KB cells are inhibited when parachuted on sheets of amnion cells. They are developing; they are dividing. But they do not overlap. In the control experiments, where we used normal human kidney cells, there was intermixing.

The last essential point is that the movement of the normal cells was as important in this kind of intermixing of normal and malignant cells as the movement of the malignant cells. This is a very important point. This situation, where the mobility of normal cells is an essential factor in the invasion, actually also happens *in vivo* in every repair process where the fixed elements of the normal tissue are mobilized.

Dr. Abercrombie: Dr. Barski has mentioned the nonparalysis of ruffled membranes on contact among certain cells. This, I think, underlines my present feeling of uneasiness as to whether the paralysis that one observes with normal fibroblasts is really an essential part of the inhibition that stops them from monolayering. One fails to get this paralysis among a number of other kinds of cells, including Sarcoma 180, which has quite a degree of monolayering, but shows none of this paralysis, and among wandering cells, polymorphs and macrophages, which have an extremely strong tendency to monolayer, but show no paralysis of membrane. The whole business of paralysis of membrane may turn out to be a red herring altogether. This must wait for further investigation.

Regarding Dr. Barski's point about the movement of normal cells, I would have liked to have said something about that during the talk. This, certainly, we have found. Invasion is mutual. Of course, there are quite a number of tumors which are very inhibitory to fibroblasts and upset them extremely. The amount that a fibroblast population can invade a malignant population depends on the degree of this upset.

Sarcoma cells, on the contrary, are quite commonly encouraged by the presence of fibroblasts. This is a diffusable interaction in most cases, something rather different from contact.

But apart from that, certainly, the failure of contact inhibition is a mutual matter. In these circumstances, fibroblasts are invasive.

Dr. Leighton: I support the point made by Dr. Barski. About 6 years ago, we described the invasion by HeLa and other human cell lines of cultures of normal connective tissue in sponge-matrix systems. We observed most extensive infiltration of connective tissue by tumor cells where fibroblasts were oriented radially to the site of the tumor inoculum.

In addition to the invasion of the normal tissue by tumor cells, we saw infiltrative migration by connective tissue cells into the tumor inoculum. In other words, in our 3-dimensional system, a reciprocal invasion was found, especially during the early stages of these combinations.

We observed an additional indication of the active role that connective tissue cells may play in the local spread of cancer with time-lapse studies of these combinations. Occasionally, a single or a small clump of tumor cells was seen to be firmly adherent to a fibroblast for prolonged periods. During such intervals, the fibroblast moved across the microscopic field carrying the tumor cells with it. Our observations indicate that the migratory activity of normal cells as well as of tumor cells is of importance when tumor invasion is examined *in vitro*. I agree with Dr. Barski that it may have its counterpart in the intact animal.

With regard to the state of contact inhibition that may exist in the normal connective tissue in the body, of great importance are the recent observations of several

investigators that environmental changes may alter or abolish contact inhibition of normal cells. Perhaps an infiltrating carcinoma discharges metabolic products in its vicinity that release the surrounding uninvolved normal tissue from contact inhibition. Such noninhibited stromal cells would be free to respond to other cues from the tumor affecting their migratory reactions and spatial arrangements in a manner that would further the infiltrative progress of the tumor.

Dr. Moskowitz: I would like to speak about two aspects of this. First, we have studied aggregation of established strains of cells using one strain of cell that has been in culture now for approximately 11 years, a monkey kidney cell isolated by Dr. Hubble. We get aggregation in practically identical fashion insofar as kinetics go with cells from 10-day embryos. When these cells are put in a tube, they attach to glass almost immediately. When they are rotated at a relatively rapid rate, they do not attach and aggregate in the fashion that we see with embryonic cells. If we add puromycin to the culture, the cells immediately attach to the glass, but the puromycin inhibits the aggregation. At any time up to 24 hours, which is as far as we have gone, if we remove the puromycin and put in fresh media, the cells start their aggregation pattern again. This suggests that, for the aggregation to occur, some extracellular material of Dr. Moscona has to be made there, but this is not necessary for the cell attachment. One could think of the cell attachment as being due to some charge on the surface, of course, for the immediate attachment. If one washes the puromycin off, after a period of about 3 or 4 hours, the cells no longer attach to glass, but, as I said, they can continue aggregating. This can perhaps explain why such cells do not attach to glass readily and round up.

I would also like to comment on what Dr. Barski just said about contact inhibition and clumping—that is, surface attachment. We also are working with a strain of L cells, and we are studying the effect of different fractions of peptone on the growth of these cells. These cells are maintained in protein-free medium with peptone. With one fraction of peptone, we get a beautiful monolayer with what appears to be excellent contact inhibition. With another fraction of peptone, with the very same inoculum, we have practically complete lack of contact inhibition, and this clumping occurs within 24 hours. In this instance, we see different gradations with the different fractions. But we see variation in the behavior of the same strain of cells, depending upon the nutrient there. I would like to think that this clumping phenomenon is due to the production of this extracellular material.

One could speculate that in one fraction this extracellular material is not made and, therefore, it remains as a monolayer whereas, in the other fraction, this extracellular material is encouraged to be made.

Dr. Moscona: In reference to Dr. Moskowitz's statement, surely, cells treated with trypsin—I take it that you dissociate yourselves with trypsin—have their surface affected. We have just talked about it. There may be other damage points. Therefore, this is why they may not adhere to each other, but the damage may not be sufficient for preventing them to stick to glass.

It is very possible that this is due to ionogenic groups on the cell surface. Although I am not a physicist, I do know that charges are not metaphysical. There is always a charge-bearing group. There is something which is a charge carrier.

In other words, the fact that cells have a surface charge doesn't mean that the charges in themselves are the important factor in their behavior, but it could mean that these charges are carried by charge-carrying groups—essentially the mechanism which mediates the effect of these charges in terms of adhesion and cohesion of cells.

I want to corroborate your puromycin effects. Three years ago, we showed that inhibition of protein synthesis at the level of RNA control inhibits aggregation of dissociated cells. We also showed that inhibition of RNA synthesis by actinomycin under very controlled conditions inhibits aggregation or after a lag period consider-

ably longer than that which carries the inhibition by puromycin. This has led us to ask whether the production of these cell surface materials that might be involved in aggregation is not controlled genomically as most products in cells are.

Dr. Moskowitz: We get the same results with mechanically dissociated cells without the use of trypsin.

Dr. Moscona: Mechanically dissociated cells are also dissociated—meaning the damage has been inflicted upon the cell surface.

Also, mechanically dissociated cells, as we know now from the work of the group at the Strangeways Research Laboratory, may undergo internal changes, activation of internal enzymes, which may affect the cell surface. This possibility cannot be excluded.

Trypsin-dissociated cells have been compared in terms of fine structure and metabolic aspects with Versene-dissociated and tetraphenyl boron-dissociated cells by Harris. He found in terms of fine structure differences that, for instance, Versene inflicted more irreversible damage on these cells than trypsin.

Dr. Moskowitz: Your comments relate to the dissociation of tissues; we are working with monolayer cultures, and the cells from such a culture are relatively easily dispersed mechanically.

Dr. Sano: I would like to point out that I was interested in the Sarcoma 180 lacking contact inhibition which Dr. Abercombie reported. However, in human tumors which are highly malignant, such as glioblastoma multiforme, I have movies on file which show the tumor cells to have very marked contact inhibition and extremely fast growth. Besides that, they are interesting because these cells do not divide unless they have contact with other cells of the tumor, the cytoplasmic contact.

Another interesting finding, which bears further investigation, is the proteolytic action of these tumors. Because in section, these tumors resemble very much what you would get in tissue culture because the brain substances have actually been markedly changed chemically. Of course, these experiments were done many years ago and so have not been confirmed chemically.

Dr. Abercombie: From my point of view, the really interesting point is: What is this contact inhibition shown toward in this tumor? Is it shown toward the kind of cells which they normally invade or are you referring to contact inhibition between the tumor cells?

Dr. Sano: Between the tumor cells.

Dr. Abercombie: I am not at all confident that this is necessarily correlated with the contact inhibition that the tumor would show to the cells they normally invade. From my point of view, this is the crucial thing that one has to check.

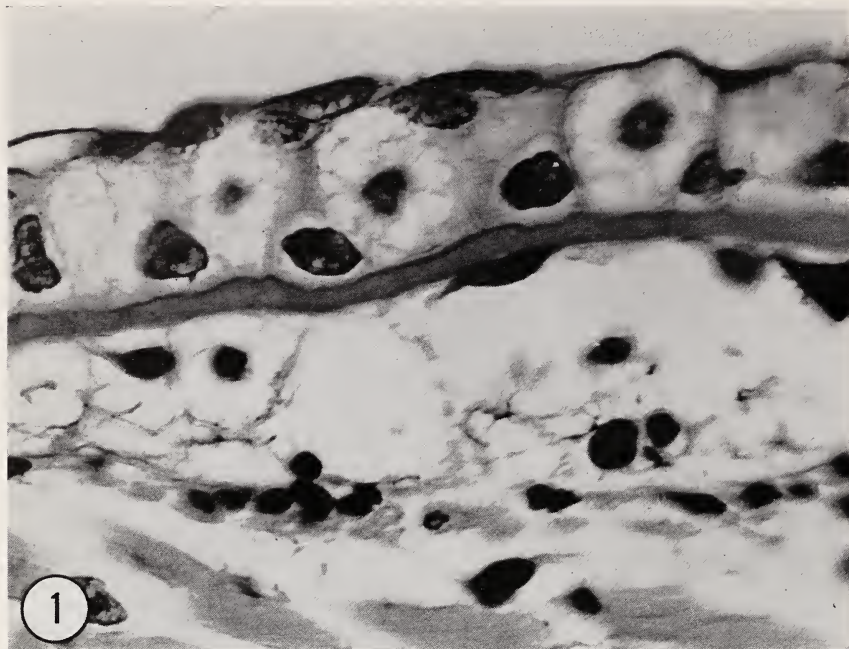


FIGURE 1.—Cross section of larval urodele epidermis, showing various cell types attached to basement lamella. $\times 600$

Figures 1, 2, and 3 originally appeared in the *Journal of Experimental Zoology* (vol. 128, pp. 13-28, 1955) in an article by J. W. Lash entitled "Studies on wound closure in urodeles."

Figures 4 and 5 have not been published previously, but the work was referred to in an article by J. W. Lash entitled "Tissue interaction and specific metabolic responses: Chondrogenic induction and differentiation." In "Cytodifferentiation and Macromolecular Synthesis" (Locke, M., ed.). New York, Academic Press Inc., 1963.



FIGURE 2.—Migrating epithelial sheet after wounding. Migration in direction of *arrow*. Movement ceases when epithelial sheet contacts epithelium moving in from opposite site of wound. $\times 500$

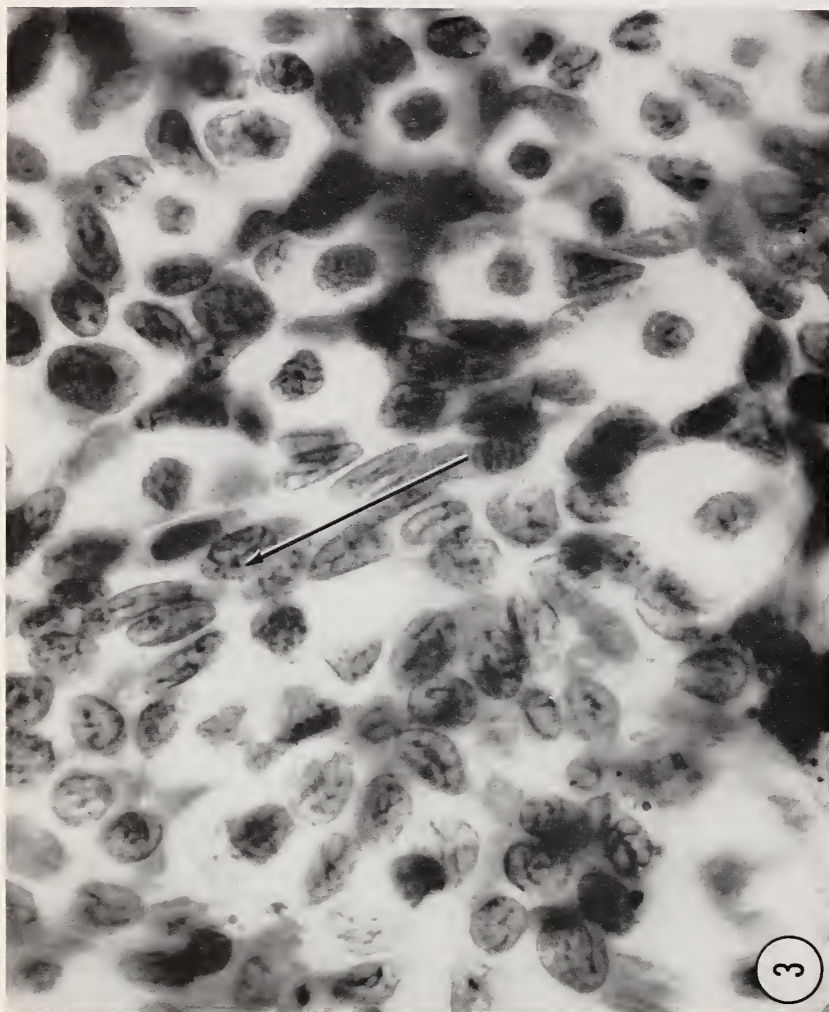


FIGURE 3.—Portion of migrating epithelial sheet showing clusters of small cells oriented in direction of epithelial movement (*arrow*). $\times 600$

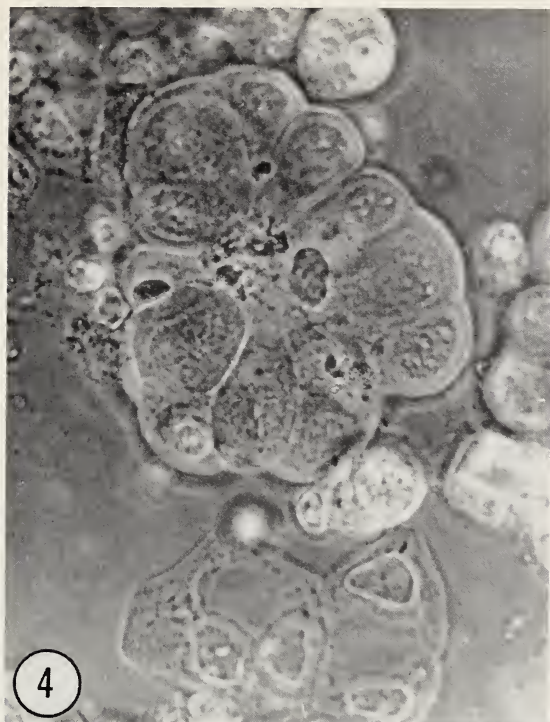
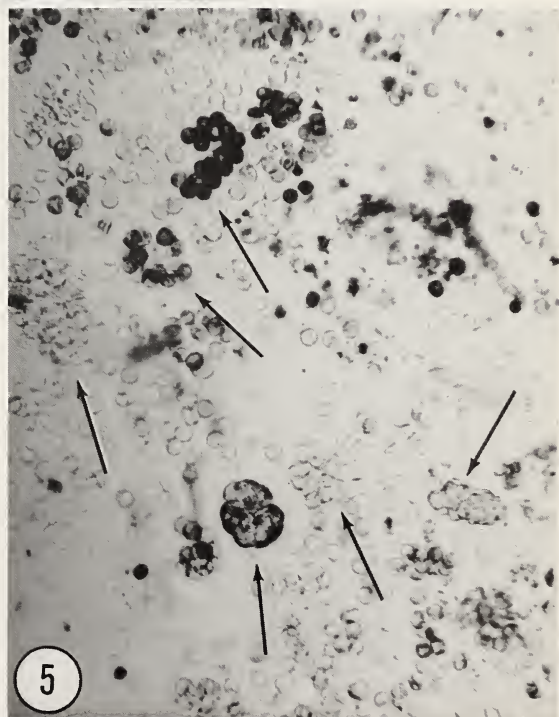


FIGURE 4.—An aggregate of epithelial cells, 30 minutes after dissociation. $\times 800$

FIGURE 5.—Aggregates of homogeneous cell types. The epidermal cells aggregate only with their own cell type. Arrows show various types of homogeneous clusters, viz., various types of pigment cells and epidermal cells. $\times 100$



Mechanisms of Organogenetic Tissue Interaction¹

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SUMMARY

Progress during the past decade toward understanding mechanisms of embryonic inductive tissue interaction is outlined and evaluated. Basic properties of such interactions frequently include reciprocity, some degree of specificity, measurable time span and multiplicity of involved factors. The factors in some instances are separable from cells, but

their mobility in active form is limited in varying degree. Circumstantial evidence suggests that macromolecular complexing at inductive interfaces may be an important factor in embryonic induction. A model along these lines is proposed for investigation.—*Nat Cancer Inst Monogr* 26: 279–299, 1967.

I SHALL FOCUS on progress made in the past decade toward understanding mechanisms of interaction between components of developing organ rudiments. The importance of such interactions was generally recognized long before the beginning of the decade, in fact as early as the end of the first quarter of the century (1). The phenomenon, which came to be known as embryonic induction, was the subject of intense interest as the second quarter-century began, and its universal importance in organogenesis became manifest. Progress in establishing its mechanisms remained slow, however, because of both conceptual and methodological difficulties. During the past decade these difficulties have increasingly been resolved—in part through exploitation of culture techniques (2). The stage is now set for major advances in the decade to come. In what follows I shall deal primarily with recent work which seems to me to justify this optimistic appraisal. I shall make no effort to be exhaustive in coverage of the literature, nor to confine myself entirely to data obtained *in vitro*. Nonetheless, because the subject so clearly demands and is il-

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

luminated by approaches *in vitro*, I believe it is entirely appropriately reviewed as part of a Decennial Review of Tissue Culture.

BASIC PROCEDURES

General procedures for culture of developing organ rudiments, earlier initiated and refined at the Strangeways Laboratory by Fell and her associates, have been modified and elaborated to permit experimental approach to virtually any higher vertebrate rudiment (3). For example, the technique has rendered mammalian development at least as advantageous for study as the more traditional lower vertebrate and invertebrate material. Various modifications of the basic procedures have opened developing rudiments to special methods of observation (4). Combinations of normally separated organs have been made *in vitro* (5), and organ responses to experimentally imposed reagents and conditions have been studied (6).

Organ rudiments can be separated into their components and these cultured either separately or on recombination (2). Failure of components to continue development in isolation, and renewal of development on recombination, have been used as a fundamental test for an essential developmental interaction (7). By this test all rudiments that form from two or more tissues brought secondarily into association show dependence between these tissues for continuance of development. Moreover, where the tissue components each undergo a developmental change following their association, the interaction is frequently reciprocal—each depends upon the other for continued development. The period of dependency, alternatively called the period of induction, is of measurable duration. Components kept too long apart lose the ability to interact (8) and components separated following a sufficient period of association can develop independently (9). Components may be reassembled on either side of membranes of suitable porosity and interactions will continue (10). By increasing the thickness of the intervening membranes, or altering their porosity, information can be obtained on the mobility of the effective materials (11). Exchanges of materials can be followed by radioisotopic labeling and autoradiography (12). In a few instances active cell-free extracts have been prepared (13,14).

The availability and results of these procedures afford a number of convenient experimental systems for application of sophisticated analytical techniques to the problem of mechanism. All interactions are not expected to proceed by the same mechanism, nor will any one system provide answers to all questions. Rather, discriminating selection and comparative study of suitable systems are likely to be the optimum approach. I shall take up, with these considerations in mind, the general features of

organogenetic tissue interactions relevant to questions of mechanism. For simplicity, I shall concentrate on two-component systems, though many organogenetic courses involve more than two (15).

BASIC PROPERTIES

Reciprocity.—Early demonstrations of developmental dependency came from instances where the consequences of separation of components were morphologically obvious. Because the consequences are frequently much more obvious in one component than in the other, therefore, induction was first observed and conceived as a one-way street with one component acting as inducer, the other as responder (1). It now seems likely that apparent one-way inductions are special cases of developmental interaction, and that both components can be shown to be altered if suitable detectors are applied. This is the case in such organs as the kidney (16) and limb (17), and recently the point has been emphasized in the development of the tooth bud (18) and liver (19). In the latter, the primary epithelial hepatic bud of the chick embryo fails to develop further as liver when isolated from its surrounding mesenchyme. Recombined with hepatic mesenchyme it differentiates into hepatic cords and then into hepatocytes.

Hepatic epithelium thus depends upon mesenchyme for continued development. However, hepatic mesenchyme behaves differently in the presence or absence of hepatic epithelium. In the absence of epithelium, the mesenchyme is organized into lacunae resembling liver sinusoids. When epithelium migrates into such mesenchyme, the mesenchyme is transformed into endothelium, which in turn undergoes hematopoiesis. Thus the course of mesenchymal development depends upon the presence or absence of epithelium. Typical liver development results from *reciprocal interaction* between two component tissues leading to alteration of the developmental course of *both*. The two-way, rather than one-way, character of the interaction obviously has consequences for considerations of mechanism.

Specificity.—The interaction of organogenetic components shows some degree of specificity, *i.e.*, there is limitation in the range of tissues that can replace the normal component in eliciting a given developmental response. For example, specificity is high in the induction of mouse salivary epithelial morphogenesis, which appears to be promoted only by “condensed” mesenchyme of a salivary rudiment (8). [Interestingly, active mesenchyme can be obtained from the parotid salivary rudiment of the mouse (20), or from the salivary rudiment of the chick (21). It appears to be the type of rudiment rather than topographic location or species derivation which is important.] On the other hand, specificity is low in the induction of the growth and differentiation of mouse pancreatic epithelium, whose

development *in vitro* is promoted by any embryonic mesenchyme tested so far (22). Other epithelia are of intermediate specificity. Lung epithelium, for example, fails to continue development when combined with somites, mesonephric, or chorioallantoic mesenchyme, but does continue branching and some differentiation in metanephrogenic mesenchyme (23). Lung epithelium, however, develops best in lung mesenchyme. Similarly, thymus epithelium develops best in its own mesenchyme, but also progresses in lung, salivary, and metanephrogenic mesenchyme (24). Unlike these two epithelia, pancreatic epithelium is promoted in its growth and differentiation even more strongly by salivary mesenchyme than by its own pancreatic mesenchyme (22).

Although these variations cannot yet be fully explained, certain preliminary generalizations may be made in considering mechanisms. First, the general response of pancreatic epithelium to all mesenchymes suggests that they produce a common factor or factors not produced by epithelia or other tissues tested (mesenchyme common or MS-factor).

Second, some, or perhaps all, mesenchymes produce an additional factor or factors peculiar to a particular mesenchyme. Salivary mesenchyme, for example, has a property essential for salivary epithelial morphogenesis not found elsewhere. Factors specific to particular mesenchymes may be referred to as mesenchyme-specific or MS-factors. MS-factors may be not only differentially produced by various mesenchymes—and differentially required by various epithelia—but may partially control the nature of epithelial responses. Pancreatic and thymic epithelium, for example, has different branching patterns in different mesenchymes (22, 24). More strikingly, however, embryonic epidermis is reported to develop differently depending on the source of its mesenchymal association (25). On limb mesenchyme it keratinizes normally. On gizzard mesenchyme it not only fails to keratinize but becomes mucus-secreting and even ciliated. On proventricular mesenchyme it first behaves as it does on gizzard mesenchyme, but then shifts back to keratinization. MS-factors, therefore, may not only be differentially produced and required but may contain developmentally significant information about control of decisions between alternative developmental courses.

Third, three grades of epithelial behavior with respect to mesenchyme may be distinguished: 1) spreading to form thin sheets with little or no cytodifferentiation; 2) rounding to form spheroidal compact masses undergoing keratinization or other "simple" cytodifferentiation; 3) organized and characteristic ramification with accompanying "complex" cytodifferentiation. Generally, behavior of the first grade occurs on substrates in the absence of mesenchyme, behavior of the second grade in the presence of MC-factors, and behavior of the third grade in the additional presence of MS-factors. Epithelia appear to differ in the relative importance of MC- and MS-factors for continued normal development (*e.g.*, sali-

vary and pancreatic epithelia). MS-factors may be more important at earlier stages and less important at later ones (26).

Fourth, various epithelia have an apparently common effect on mesenchyme, in that they tend to aggregate mesenchymal cells in greater density in their immediate vicinity. This is manifested in the "condensed" mesenchyme characteristically associated with epithelial rudiments (8). It is not clear whether condensed mesenchymes are more than superficially alike, nor whether the epithelial factors that induce them are identical. Various epithelia can have different effects, *e.g.*, the apical ridge epithelium versus proximal epithelium in the developing limb (17). Further study is necessary to establish whether a distinction should be made between epithelium-common (EC) and epithelium-specific (ES) factors in the same sense as the MC- and MS-factors are defined above. Answers to these and related questions should result from systematic investigation of the interactive properties of well-chosen sets of components in their various combinations.

Multiplicity of interactive factors.—The nature and number of the interacting factors involved in a given inductive process are unknown. Accumulating information suggests, however, that earlier efforts at explanation through transfer of single "organizer" substances, morphogenetic materials, or information-rich, quasi-genetic molecules represented serious oversimplification. The distinction made between common and specific factors, and the probable differences in the specific effects exerted by mesenchymes from various rudiments suggest a multiplicity of mesenchymal factors. Further, there is evidence that physical substrate also plays a role. For example (27), the basal layer of chick embryonic epidermis normally is columnar and mitotically active. Orientation regarding the epidermal-dermal interface, as well as DNA-synthetic activity, are lost when the epidermis is separated from the dermis. Maintenance or recovery of the active state occurs on recombination with dermis, or in the presence of macromolecular factors in embryo juice. The important point here is that the embryo-juice factor(s) is effective only if the epidermis touches a suitable physical substrate. The importance of substrate contact was suggested by the difference in behavior of epidermis placed on a surface in normal or reversed orientation. It was strikingly demonstrated when the epidermis was spread on a Millipore filter membrane with holes 0.15 mm in diameter. Despite the uniform presence of effective nutritional factors, the basal layer was oriented and incorporated tritiated thymidine only where it was in contact with the filter, and was unorientated and did not incorporate thymidine over the holes. Collagen gels and frozen-thawed dermis were at least as effective as substrates, but lens paper or 100,000 $\times$ g pellets from embryo juice were not. It is important to understand the mechanism of this striking substrate effect.

Mobility of inductively effective substances.—The importance of a physical substrate recalls that for many years “contact” reactions were assumed to underlie embryonic inductions (28). We now know that, given suitable conditions in the interspace, inductive interactions can occur across measurable distances. This is true not only of salivary and kidney rudiments on which the demonstration was first made (29) but of a number of other developmental interactions as well. Spinal cord or notochord, can interact with somites across a membrane filter to produce cartilage (2, 30–32); pancreatic (22), thymic (24), and mammary (33) epithelia can similarly interact with mesenchyme; and apical ridge epithelium can interact with underlying limb mesoderm across a membrane (34). So, too, can ameloblastic and odontoblastic components of a tooth germ (18) and optic vesicles and lentogenic epithelium (35), the interaction which earlier provided much of the evidence for a strict contact requirement. It is reported (36) that amphibian primary induction also can occur across a membrane filter. These observations confirm that materials of some degree of mobility are involved in most, if not all, embryonic inductions.

There may be variation in the range of, and conditions for, mobility of the materials effective in different interactions. The amount of available information is greatest for the interaction of dorsal spinal cord and metanephrogenic mesenchyme (11). When the intervening membrane has an average pore diameter of $0.5\ \mu$ or greater, interaction is at maximum intensity over distances up to about $30\ \mu$, declines up to 60 – $80\ \mu$, and is absent at greater distances. Membranes whose average pore diameter is about $0.1\ \mu$ distinctly limit the distance of effective interaction—weak to moderate reactions occurring at 20 – $25\ \mu$ but not beyond. Cellophane cuts off all interaction, but when a small hole is made in it, interaction occurs through the fluid-filled hole and the response is localized immediately over it. Large-molecular labeled material, produced by dorsal spinal cord exposed to tritiated amino acids, shows a mobility and distribution entirely in accord with this biological activity (12).

More limited studies of induction by ventral spinal cord and notochord of cartilage in somite mesoderm (32) show somewhat attenuated effects of both at $50\ \mu$, but no effect of ventral cord at $75\ \mu$. Interestingly, attenuation of the notochord effect either by increasing the distance, or by preculturing the cord, tends to alter the morphogenetic response in the direction of that characteristically produced by ventral cord. Instead of “partial” nodules, with oriented and elongated cells closely applied to the notochord or filter surface, “complete” nodules are produced with surrounding perichondrial sheath. Conversely, when situations are arranged that might be expected to concentrate substances emanating from ventral cord, nodules occur more often. Additionally, under these circumstances cartilage nodules frequently appear with cells oriented along a filter surface *at a distance* from the inductive source. This is reminiscent of the importance

of a physical substrate in the skin experiments (27) referred to earlier. It reinforces the possibility that surfaces and interfaces, as well as materials, play an important part in organogenetic processes.

Dermal effects on epidermis are weak but definite at distances of 150 μ with membranes rated at 0.45 μ average pore diameter (37). This is a greater distance than recorded for spinal cord effects on metanephrogenic mesenchyme. However, as with spinal cord, membranes of 150 μ thickness with 0.1 μ average pore diameter fail to transmit the dermal effects, while 2.5 μ thick membranes of similar porosity do transmit the effects. The dermal factor seems to move over greater range (or to be effective in lower amounts) than the spinal cord factor, but its mobility is limited by pores of similar size.

Time course of the interactive process.—The time course of the reaction between components of cultured rudiments, particularly when they are interacting across a membrane filter, is readily analyzable since the components can be separated or recombined at will. Somite chondrogenesis, metanephric tubulogenesis, and pancreatic differentiation, among others, have been closely examined from this standpoint. In each instance a period of some hours is required before the responding component is sufficiently established on its new course to become independent of the inducing component. Beyond this step, the response appears to be gradually stabilized over a considerable period and probably through several stages. Mouse metanephrogenic mesenchyme is a useful example for investigation and comparison.

Kidney.—The ureteric bud is normally the inducer of tubules in nephrogenic mesenchyme in kidney formation, but it can be replaced by dorsal spinal cord (16). The kinetics of reaction between spinal cord and metanephrogenic mesenchyme has been examined in some detail (16, 38). Morphologically, tubule formation is discernible after about 24 hours, both in living cultures and in stained sections of fixed cultures. Early "condensed aggregates" of mesenchyme cells tend to form whorls which gradually transform into tubule rudiments whose peripheral cells are increasingly sharply marked off from the surrounding nontubular mesenchyme. Radial elongation of the peripheral cells occurs, accompanied by formation of a basement membrane. By 40–50 hours the cells are distinctly epithelial and a lumen is apparent in the tubule. Beyond this, continued growth leads to the typical S-shaped, and then coiled, tubule which eventuates in a characteristic secretory loop and glomerulus.

If the spinal cord is removed prior to 24 hours no definitive tubules are produced (20). If the cord is removed after about 30 hours some tubules nearly always form. In this approximately 6-hour period, therefore, a critical change occurs which stabilizes the first forming tubules. Without continued inductive stimulus, the stabilized tubule is abnormally small (20), but continued stimulus leads to formation of additional tubules. The

action of the cord, therefore, is a continuing one beyond the minimum period necessary for the first stable effect.

The basis for the latent period of some 30 hours is not entirely known. It could involve: a) "production time" by the source tissue; b) "transit time" of the active material between source and responding tissue; c) "response time" of the reacting tissue itself. Probably all three of these are factors. During the first 6 hours large molecular materials already are moving between the interacting tissues (39). These materials are alcohol-formalin-precipitable, trypsin-digestible, and label with tritiated amino acids. They conform in their distribution in a membrane filter with the region of forming tubule rudiments. As judged by incorporation of label transferred from inductive source to responding tissue, significant amounts of materials of this sort have arrived in the mesenchyme by 6 hours. It is not, however, until some 18–24 hours later that a distinct response is detectable by several criteria. Morphologically, tubule rudiments are detectable as condensations. Metabolically, uridine incorporation—reported to decline during the first 2 hours in culture—begins again to increase and is detectable in ribosomal and messenger RNA fractions (40). Possibly in correlation, tubule formation loses its sensitivity to actinomycin D inhibition (41). The observations conform to expectations if a new line of differentiation is established at about 24 hours by issuance of a new set of instructions from the genome. Detailed study of this period from 12–24 hours clearly is indicated as an approach to the mechanisms by which products of one tissue promote a new developmental course in another.

Initial stabilization far from completes the developmental course. The forming rudiments persist after 30 hours of exposure to spinal cord, but even after 48 hours—upon dissociation to the cell level—they cannot reform tubules unless spinal cord is again present (42). Still later, kidney epithelium acquires a new property which, without an additional spinal cord contribution, enables it to reconstitute tubules following dissociation (43). Since specific reaggregation appears to depend upon surface-associated materials, the altered property may involve these materials.

It is interesting in this connection that tubule formation alters susceptibility to viral infection (44). Immunofluorescence data indicate that polyoma virus antigen is synthesized in metanephrogenic mesenchyme cells but not in those of the pretubular condensations or mature tubules. Whether the altered sensitivity to virus relates to penetrability or capacity to synthesize virus is not clear. More precise definition of the relationship between these events, and of seemingly concurrent enzymatic changes (45), may reveal much about both the control of differentiative events and the conditions required for virus infectivity and growth.

Pancreas.—The kinetics of interaction between mouse embryonic pancreatic epithelium and mesenchyme was summarized recently (46) and needs only brief review. The readily visible and dissectable rudiment at 11 days of embryonic age is the product of earlier formative processes

whose nature is not understood. Even before the visible rudiment appears, the developing gut and associated tissues, isolated under suitable conditions *in vitro*, give rise to a pancreas (26). Epithelium of this and somewhat later stages does not by itself undergo characteristic pancreatic differentiation, though it will on recombination with neighboring mesenchyme. By 11 days a mesenchymal requirement for epithelial differentiation still exists, but it can be met by mesenchyme from any source. If such mesenchyme is present for at least 30 hours, the epithelium can continue to develop on its own, providing it remains a compact mass. If the mass is fragmented and individual pieces spread thin on the substrate, characteristic differentiation is inhibited. If the fragmentation is delayed until 48 hours of exposure to mesenchyme, the procedure no longer inhibits differentiation. By about this time or soon after, also, some cells have become insensitive to actinomycin D, *i.e.*, they continue zymogen synthesis at concentrations of the drug which earlier would block the appearance of new specialized syntheses. Still later, frank zymogen synthesis is detectable both ultrastructurally and by determination of enzymatic activity.

Cartilage.—Studies of cartilage differentiation following interaction of somites and ventral spinal cord (37) show similarities with kidney and pancreas, and provide some additional information. Somites have an earlier developmental history which has conferred predispositions for differentiative behavior. At the most studied stages—from 15–30 somite embryos of chick and mouse—the probability of their forming cartilage on isolation *in vitro* is increasing rapidly. If the somites are taken from the earlier stages, the low probability of cartilage formation is considerably increased in the presence of ventral spinal cord, notochord, or other cartilage whose cells are undergoing hypertrophy and matrix synthesis (31). Eight to 10 hours of exposure to ventral cord is sufficient to produce cartilage matrix within 3–4 days. The intervening so-called “latent period” turns out, however, not to be entirely “silent” so far as the occurrence of significant events anticipatory of frank chondrogenesis is concerned. Both sulphate incorporation and sulphate-activating enzyme activity are detectable well before frank chondrogenesis sets in. Whether this represents a “basal condition” characteristic of many tissues or an essential prelude to specific synthesis is not fully established.

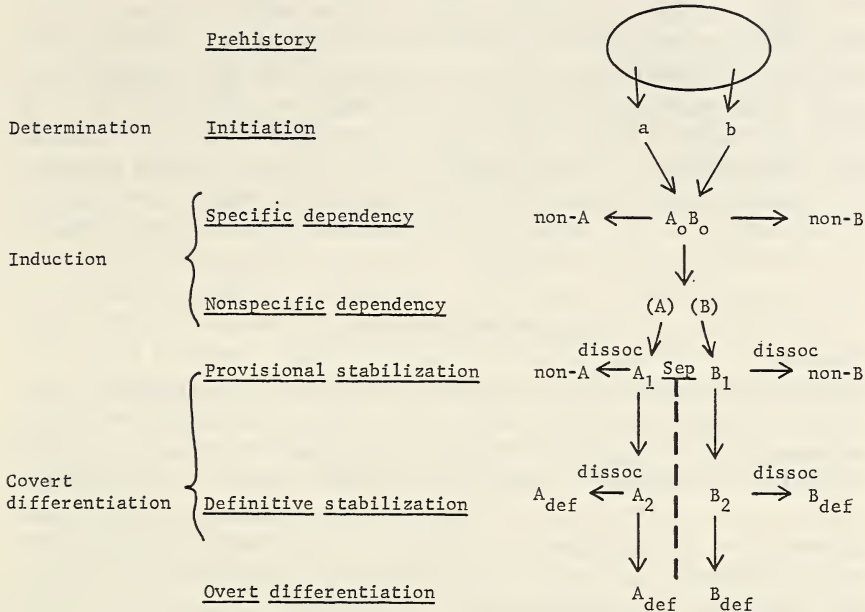
From these instances, and studies of the lens (15) which has been analyzed largely *in vivo* or in grafts, it appears useful to distinguish the following phases in organogenetic tissue interaction (text-fig. 1): 1) An *initiator*y phase during which processes and events occur within an embryonic region that give rise to components and bring them into association. During this phase, formation of the rudiment depends on conditions in its “surround,” the rudiment arising within a region (field) of the embryo larger than itself if the region is not too disturbed. The nature of the stabilizing conditions (determination) is poorly understood, but these conditions contribute some degree of bias to the developmental possibilities of

emerging components. 2) A phase of *specific dependency* during which developmental progression occurs in the associated components only if they remain in their normal tissue-typical association. The variation in the degree of requirement for specificity in different rudiments is without any particular rationale at the moment. 3) A phase of nonspecific dependency during which progression occurs in the components if the essential normal component is replaced by rather generally distributed substituents, *e.g.*, all mesenchyme. The two dependency phases distinguished here make up the "inductive" phase. 4) A phase of *provisional stabilization* during which progression of differentiation occurs in one or both components in the absence of its previously required associate—providing the tissue is maintained intact with undisturbed cell relationships. 5) A phase of *definitive stabilization* when differentiation is not yet overt by ordinary criteria for measuring it, but progression to full expression occurs under any circumstance which allows the cells to remain viable. 6) A phase of *overt differentiation* in terms of specialized product synthesis and/or special morphology.

These six phases are not necessarily distinct or invariably present in every organogenetic interaction. It is not clear, for example, whether specific dependency necessarily precedes nonspecific and whether definitive stabilization exists in the absence of any specialized synthesis. Nonetheless, it seems important to distinguish these phases to emphasize not only that organogenetic processes go on over a considerable period, but that they involve changes of rather different kinds and, presumably, of mechanisms. Conceptually, the recognition that organogenetic interaction is not an instantaneous *event* but a complex *process* going on for an extended time is the major advance stemming from studies of the past decade. Accordingly, models for the mechanisms involved no longer seem attractive if they suggest that one component alters the other in a single-shot transfer of information.

Considerations of mechanism.—It is fair to say that, though there is no instance of organogenetic tissue interaction in which the underlying mechanism is understood, we now have enough information to write a set of probable specifications for the mechanism as a guide to further efforts.

In contrast to a decade ago, we now deduce that materials separable from living cells have an important function in embryonic tissue interaction, though cell surfaces may also play a role. The materials probably are predominantly large-molecular, since the activity can pass Millipore filter membranes but not cellophane; they incorporate label introduced as amino acids or nucleosides, and very likely embody considerable specificity. In one instance in which cell-free materials essentially duplicate the action of a normal component the activity is sedimentable at $100,000 \times g$ and very difficult to solubilize (47). This extract can replace mesenchyme in stimulating the growth and differentiation of pancreatic epithelium (13, 46), and furthers columnar orientation and proliferation of epidermal basal



TEXT-FIGURE 1.—Time course of organogenetic interaction, *a*, *b* = precursors for components; *A*, *B*, *A₀*, *B₀* = first identifiable components; *A₁*, *B₁*, *A₂*, *B₂*, *A_{def}*, *B_{def}* = successive states of *A*, *B* leading to the definitively characterized differentiated state of each. Arrows indicate progression in time; *Sep* = separation of components; *dissoc* = dissociation to cell level followed by reaggregation.

cells (27). In the latter case the growth-stimulating effect is further enhanced by the presence of a protein extractable from mouse salivary glands, though only when the basal cells are in contact with a suitable substratum. In another instance, activity for cartilage promotion in somites has been obtained in soluble form, but its precise characteristics are yet to be specified (37).

There is reason to assume that a multiplicity of substances is involved in organogenetic interaction, possibly even in a single interaction. Whatever the size of the active molecules, their mobility in active form is usually somewhat restricted. Restricted mobility, of course, makes sense in a control system which must provide localized effects in the small but highly heterogeneous embryo. The restricted mobility, however, need not be of the molecule itself, since inactivating mechanisms can be conceived which would fit existing data. Whatever the mechanism, there is never much activity, and this may account both for the difficulty of extraction and the continuous production of active material which probably occurs. This suggests that successful efforts to accumulate activity may have to provide for prolonged collection from living tissues under conditions which stabilize materials through fractionation even as they are produced.

In this connection, there is growing evidence—albeit still incomplete—that substances normally associated with the cell surface and accumulating at tissue interfaces may play an important role. The suggestion was made some years ago (2) that new molecular complexes arising in the interspace between interacting tissues might provide a key to the new behaviors of tissues on either side. Since then evidence (48) has supported the hypothesis that a periodic acid-Schiff-positive mucoid material, associated with epidermis following its incomplete separation from dermis, maintains the columnar orientation of the basal epidermal cells. This same kind of orientation of basal cells has been shown to depend not only on suitable nutritional factors but on contact with a suitable interface (27). Conversely, dissolution of the basement membrane has been reported to lead to epidermal-dermal interactions essential to formation of a supernumerary limb in amphibian embryos (49). These and other observations, together with the specifications for organogenetic mechanisms outlined here, attract attention to interfacial materials in relation to the developmental events progressing on either side. Conspicuous among interfacial materials are those involved in formation of histologically defined basement membranes. At least several components defined ultrastructurally and chemically may be involved in the histological basement membrane (50). The source of these components, the nature of their interaction, and their relation to morphogenetic events are receiving increasing attention.

There is much evidence that epithelia produce at least one mucoid component which is detectable ultrastructurally, immunochemically, and histochemically (50, 51). Ultrastructurally, this component corresponds to what has been called epithelial basement membrane or surface-associated material (SAM). The term (SAM) will be used here. The material (52) occurs as an electron-translucent zone (SAM₁) bounded by an electron-dense line—beyond which is additional electron-translucent material (SAM₂) of varying thickness and structure. Virtually all this material is removed by trypsin-pancreatin and can be reconstituted by epithelia incubated in the absence of associated mesenchymal cells. Indicative of its epithelial origin, the material appears also at epithelio-epithelial interfaces. It probably corresponds to the substances of specific antigenicity detectable immunochemically (51). If so, it is chemically distinct from collagen, having more tyrosine and cysteine and less glycine, proline, and hydroxyproline (53). It is said to contain 2.5–3% glucosamine (54), which may account for the halo of hyaluronidase-sensitive label which appears around salivary epithelium incubated in tritiated glucosamine (55).

The evidence also is strong that epitheliomesenchymal basement membranes at least frequently include collagen as well. Collagen fibers are obvious ultrastructurally and are particularly striking in the basement lamella of amphibian skin where collagen also has been identified and measured chemically (56). The source of this collagen is still under discussion, with origin in both epithelium and mesenchyme postulated (57).

Whether or not epithelium is a source, the data certainly indicate that mesenchyme is one (58). The question is thereby raised whether some property of the epithelial surface, and possibly of its SAM, favors polymerization of soluble collagen which reaches it from mesenchyme. If so, fibrogenesis might result from complexing of mesenchymal protein with epithelial mucoid material (56). Interaction between collagen and acid mucopolysaccharides is known to occur and a schematic model for their product recently has been proposed (59).

WORKING HYPOTHESIS

The fibrogenesis of collagen at epithelial surfaces was earlier suggested as a useful model for organogenetic tissue interaction (28), as a specific example of what still earlier (2) was referred to as matrix interaction. The hypothesis was advanced at the beginning of the decade under review, and may now be restated in current form.

1. Macromolecular materials synthesized by cells, and normally remaining closely associated with their surfaces ("micro-environmental"), are important determinants of their behavior. Although the mechanisms of their effects can only be speculated upon, their "gateway" location is clearly strategic to control permeability and hence other activities of cells.

2. These materials are specific biologically and chemically and are presumably involved in reactions that require tissue recognition, *e.g.*, specific aggregation (60), viral infectivity, antigenicity, etc.

3. The materials are subject to polymerization and complexing to form fabrics whose mechanical and chemical properties play an important role in tissue architecture.

4. The materials can interact at interfaces between unlike tissues to produce new macromolecular complexes whose properties are jointly determined by the synthetic activities of the cells on the two sides. Once formed, the new complexes act back to alter the activities of the producing cells, possibly by provision of a new micro-environment which modifies the surface behavior of apposed cells. New developmental behaviors may be reciprocally induced and stabilized in this way.

A model of this kind, since it postulates a spatially extended macromolecular fabric or sheet as an intermediate in regulatory control, would provide a physicochemical basis for the kind of collective behavior of cell populations involved in morphogenesis. Not only might it yield collective and synchronized behavior but—with suitable assumptions—the kind of continuous gradients and even discontinuous periodicity, *e.g.*, segmentation and somitization, that have so far baffled students of morphogenesis. Despite its potential power as an explanatory device, the model at the

moment is based only on fragmentary support. Indications favoring it, besides those already referred to, include the correlation of invasiveness (of malignant epithelial cells into stroma) with interruptions in basement membranes (53), and the altered morphogenetic behavior of salivary epithelium subjected to collagenase (61). Efforts to test this model, or other alternatives to simple one-shot exchange, are likely to be fruitful in the next decade.

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DISCUSSION

Dr. Lash: I would like to comment on Dr. Grobstein's text-figure 1 concerning the time course of organogenetic interaction. This is a very useful scheme for defining the continuum which occurs in embryonic development, since there is seldom a single isolated occurrence that can be held responsible for the differentiation of a particular organ or structure. As Dr. Grobstein has shown, there is a continuum from the prehistory portion of his diagram, down through induction, and covert and overt differentiation. A few points may be raised, however, concerning the time course of these events. For any one tissue the sequence of events will not necessarily be the same. It is even possible that the sequence may be in slightly different order than that shown in text-figure 1. Some of the events may be abbreviated or even missing due to our present state of knowledge in the analysis of differentiation. The particular aspect I have in mind is that of connective tissue differentiation. One might say that the scheme fits pretty well the system that Dr. Grobstein has analyzed quite carefully, an epithelial system. But I think some modifications might have to be made if a scheme such as this is going to fit connective tissue differentiation.

In connective tissue systems, it seems possible that the covert differentiation might precede in order the position given here to induction, or specific and nonspecific dependency.

Evidence for this has been obtained from work done in collaboration with Dr. Giovanni Marzullo on the biosynthesis of chondroitin sulfate in chondrogenic tissues. In work to be published elsewhere, we have shown that cartilage tissues metabolize radioactive glucosamine into the precursors of the polysaccharide. These precursors, and the terminal products, may be separated and characterized by thin-layer chromatography and autoradiography. The pattern obtained on an autoradiograph is characteristic of the metabolic capabilities of the tissues tested, and a distinct pattern is obtained from cartilage tissues. We call this pattern the chondrocytic phenotype. This is an example of biochemical differentiation as contrasted to the morphological differentiation Dr. Grobstein was emphasizing. If one studies differentiation, one has to distinguish between biochemical differentiation and morphological differentiation, and what relationship one might have to the other. It is the metabolic pattern in our studies that is indicative of cartilage tissue. If similar studies are made on early embryonic tissues (at 3 days' incubation), all the tissues in the embryo possess the metabolic pattern indicative of chondroitin sulfate synthesis. The pattern is the same, but different portions of it vary in intensity. The early embryonic tissues appear to accumulate early precursors but not the final products, whereas the reverse is true for frank cartilage tissue.

This raises the possibility that in differentiation, when we induce something, we don't induce *de novo* from a biochemical viewpoint. Naturally, I think most morphological differentiation occurs *de novo*, but, in the differentiation of tissues, "induction" may mean *not* the acquisition of a new metabolic pattern, but the stabilization or enhancement of an existing one. This might relate to the predisposition or bias that Dr. Grobstein spoke of before. Possibly, the difference between the differentiated type, morphological type, and the predisposed type is the fact that the differentiated type can accumulate terminal products, whereas the predisposed type can make the terminal product, but not accumulate it.

Dr. Grobstein: The scheme shown in my text-figure 1 undoubtedly is over-generalized for any particular case. As a matter of fact, I would underline the point that in each case we must expect peculiarities. The diagram is intended to indicate the kinds of stages distinguished in various cases, and these are then ordered in their natural sequence. As Dr. Lash pointed out, many of the events which are referred to as "provisional stabilization," for example, may be occurring in certain instances at a much earlier time.

In the differentiation of cartilage, we know there is an inductive step. In the limb, however, we do not at the moment know of any inductive step in the differentiation of cartilage. We cannot distinguish a critical time when a particular tissue exerts an effect on the cartilage-forming area. Nonetheless, something comparable to what occurs in the case of vertebral cartilage must take place, since a decision is made as to the direction in which differentiation should go. I completely agree that the diagram must be modified to indicate such differences in particular situations.

As far as the distinction Dr. Lash made between events at the biochemical level and at the morphological one, we must continually strive to integrate the biochemical level and the morphological one. I know that Dr. Lash did not mean to separate these. One of our objectives must be to find out exactly how biochemical events and morphological events interact, since they are two faces of the same coin, the behavior of developing tissues.

With respect to Dr. Lash's comments on predisposition versus stabilization and underlying metabolic events, it is possible that what has been referred to as predisposition or determination (the initial steps which first stabilize the course of differentiation) may involve the beginning of product synthesis. It may, however, involve metabolism preparatory to product synthesis, or steps antecedent even to those. At the moment, we know very little about these steps. One possibility we certainly must look at is the one referred to by Dr. Lash. I would warn, however, that many synthetic pathways may be operative early in development—perhaps all possible ones to some degree—and that initiation of these may not be the problem of the developmentalist so much as what alters rates of synthesis along particular pathways as against others. In these terms, the relative performance of different pathways is much more peculiarly the developmental problem than the earliest time at which particular ones begin to act.

Dr. Zwilling: I would like to make some remarks and pose a question or two to Dr. Grobstein. In effect, this is an extension of the comments Dr. Lash has made and the discussion is going to be kept more on the biological level. Dr. Grobstein has shown very clearly the dependency between two components that is required for the continuation of morphogenesis. I would like to address myself to the question of the interdependence of histogenetic development and development of the proper form for morphogenesis. It seems that, when one reviews the development of rudiments, they can be divided roughly into two groups. In one group, largely the rudiments that Dr. Grobstein has spoken about, it is the epithelial component that gives rise to the distinctive cell types we usually associate with the structure. In the other, the mesenchymal or mesodermal component develops the typical tissue characteristics. I wonder whether morphogenesis may play different roles in the two types of situation. Let me give examples of these.

I need not mention the first category which Dr. Grobstein has discussed at some length. In the limb bud mesoderm, it is almost a difficult thing to discourage chondrogenesis from taking place in the complete absence of any association between the two components, the mesoderm and the epithelial component. One has actually to try hard to discourage the differentiation of mesoderm. In fact, Dr. Ku, in my laboratory, has demonstrated that individual cells taken from early limb bud rudiments may be cloned and still give rise to cartilage after the cloning procedure has taken place. It appears here as though the morphogenetic phase involves the ordering of a tissue which already has a strong disposition to a particular histogenetic pathway.

Now, there are intermediate cases. I consider the case of chondrogenesis again in somites as being somewhat in this category, because there is a fairly strong proclivity for this tissue to form cartilage even in the absence of association with either the nerve cord or the notochord. The latter association enhances this proclivity very greatly. And I think the questions have to be posed somewhat against this background. In the first place, is this sort of difference more apparent than real and is

it dependent merely on the conditions that exist in the two types of tissues? Is this based on the tendency of the epithelial tissue to vesiculate or spread in the absence of something that acts in a more or less mechanical way to keep these tissues cohesive and allow the histogenetic capability to be evinced?

Is it possible, in view of the prehistorical phase so nicely shown in the text-figure, that these tissues (two different types, if this is a real distinction) have different prehistories so that their sets of instruction may be different and thereby lead to different requirements for morphogenesis? I think this is where I will leave these questions. I am sure that others will come up as the discussion goes on.

Dr. Grobstein: In the most general terms, the question of Dr. Zwilling is raising is the relationship to developmental behavior of pattern among cells. Pattern is both positional and functional since it affects the kind of exchanges that take place between cells. What relationship is there between a morphological pattern and a particular cytodifferentiation involving a particular pattern of biosynthesis? We are not clear at this moment whether some new morphological pattern invariably precedes each new differentiative course, or whether, as Dr. Lash suggested earlier, the new differentiative course may begin before identifiable morphological change.

Dr. Zwilling is calling to our attention the difference in sorting behavior between two populations of cells, both of which do the same thing subsequently, as far as we know biochemically. Differences in sorting based on differences of earlier history, but with final differentiation the same, seem to me to be a subject well worth looking into. I referred to the case earlier, saying that vertebral cartilage differentiation is known to be inductive, whereas the limb cartilage arises without a particular critical association. In the case of limb there must have been something still earlier that heightened the probability that these cells would produce cartilage.

I use the expression "heighten the probability" advisedly because, although it is true that the cells of the limb have a strong predisposition to form cartilage when they are not told to do anything else, some of them form muscle when they are so instructed. There is nothing fixed about their predisposition. Nonetheless, these cells are on the way to form cartilage along a different path from those involved in vertebral cartilage. The difference in sorting behavior may be a reflection of the fact that they are following two different paths and that these somehow are reflected in their surface properties which are different at this particular time. This, in turn, would mean that it is possible to follow two different paths of instruction to the same final biosynthetic pattern.

Dr. Andrew: I believe that many aspects of the question I wanted to ask have been asked and answered in part several times. In my early career, I was in the laboratory of Dr. Ross G. Harrison. And while I was not particularly going into experimental embryology, we were pretty well saturated with organizer before we left there and had wonderful visits from Dr. Hans Stayman and others.

My question is connected with our terminology and philosophy. In the admirable talk given by Dr. Grobstein this morning, he indicated that we still are thinking somewhat in terms of mysterious or magical organizers or inducers; we might possibly think rather in terms of simply continuing the objective study of what seems to be some sort of an essential symbiotic relationship between the tissues at various stages of their development, when perhaps there is a nutritional or metabolic need for the presence of one tissue in order to bring out the full potentiality of the other.

Also, it may be important to think in terms of the potentiality of the little fragment of tissue and what it might be able to do, given certain circumstances, even without its symbiotic companion. For instance, we have thought until recently that the egg could not undergo cleavage unless it was fertilized by spermatozoon—that was the magic spark! And yet, we know now that there are other stimuli. So it may be that in the future, we will speak less of induction and less of organizers.

Dr. Rothberg: I have long had reservations about the use of the technique of trypsinization to separate epidermal from mesodermal tissue. And I have had additional reservations as a result of experiments I have done. I am trying to elucidate the structure of keratin, and I find that in epidermis freed of all soluble material trypsin in very dilute concentrations can solubilize insoluble keratin by only a single minute attack. Apparently enough peptide bonds are broken and, therefore, if you remove the trypsin with soybean inhibitor, the entire keratin molecule will apparently then unwind.

What I am relating this to, then, is the fact that I wonder whether your 26-hour dependency is due to the fact that your epidermal and mesodermal tissues have to resynthesize all the enzymes, etc., necessary that have been broken by your additional trypsin treatment. Especially riding the coattail of acid proteases and to see the action in bone development, I think this is still another thing to compete with.

Dr. Grobstein: I think you are entirely justified in having reservations that the use of trypsin may have consequences other than the ones sought. In the instances where we have used trypsin for separation, we have always validated the procedure by subsequent recombination to ensure that the tissues behave normally. I agree that, if one is looking at a time course following trypsin treatment, some part of any delay may be due to the repair, not only of trypsin effects, but of all of the effects of separation of components.

In the case of kidney, some portion of the 26-hour period of latency may involve reconstitution of whatever is involved in the separation procedure. As a matter of fact, we have evidence from the ultrastructural observations of Dr. Frances Kallman that materials removed by trypsin which lie beyond the surface take some hours to be reconstituted.

Dame Fell: This is quite an important question. I know Dr. Lash and (or) Dr. Moscona want to comment.

Dr. Lash: I want to say that we, too, have evidence that trypsin damages the cells. And in the particular instance of somite tissues after trypsinization, the specific enzyme that is required for cartilage synthesis is seriously depressed as a result of trypsinization and manipulation. And this is only to be gained later. And this, again, might be part of the latent period or the lag between induction and subsequent differentiation.

So you are right, trypsinization does do a lot of things to cells, and we really don't know how important it is.

Dr. Moscona: You know, since about 1952, when I had the privilege of being a visitor, trypsin has been introduced into cell association. There hasn't been a meeting in which this problem didn't come up.

Let's get one thing straight. Trypsin functions in dissociating tissues into cells because it is a proteolytic enzyme; it doesn't function because it has some sort of metaphysical properties. It is a tool to break proteins, and it breaks proteins. It breaks intercellular proteins, but it may also break intracellular proteins.

And there is no question—there should be no question in anybody's mind—that in doing experiments with trypsin, you have to control your system and know exactly what else trypsin does to your cells. There is no point in saying trypsin damages because, of course, it damages. It dissociates tissue with single cells. It has a damaging effect on intercellular bonds. It brings tissues in suspension. So it damages.

What else it does, as Dr. Grobstein pointed out, has to be controlled, has to be found out before you generalize. You cannot generalize that trypsin doesn't do anything to cells. Neither can you generalize that trypsin is a damaging agent. Point number one.

Dr. Pearse: Dr. Grobstein, I am very anxious at the present time about the development of pancreatic islets. What is the age of your explants and do they develop islets in the normal way and do those islets contain functioning *alpha* and *beta* cells?

Dr. Grobstein: The explants I referred to are from 11-day mouse embryos; at this stage there is a very small amount of pre-islet tissue.

Generally speaking, under the conditions we use for culture, the continued growth and differentiation of this tissue are not favorable and we get almost exclusively exocrine tissue.

In some instances, for reasons that are not altogether clear, somewhat more islet tissue is produced. We have not looked into this very carefully and cannot at the moment give you any additional information about it.



GROWTH RATE AND DIFFERENTIATED FUNCTION OF CELLS

Chairman: Professor Peter J. Gaillard, Academisch Ziekenhuis, Leiden, Holland

Speakers: Dr. Heinz Herrmann, University of Connecticut, Storrs, Connecticut
Dr. Robert DeMars, University of Wisconsin, Madison, Wisconsin

Panelists: Dr. Isaac Harary, University of California Medical Center, Los Angeles, California

Dr. Howard Holtzer, University of Pennsylvania, Philadelphia, Pennsylvania

Dr. Irwin Konigsberg, Carnegie Institute of Washington, Baltimore, Maryland

Dr. Gaillard: In talking about growth rate and differentiated cell function, growth rate, of course, is cell division and the number of cell divisions per unit of time. As far as differences in function are concerned we must realize the different levels. There are two papers, one on an aspect of nuclear differentiation and the other more or less confined to cytoplasmic differentiations.

We also have to realize that what we know about the expression of gene action not only depends on the genes and their activities but also on those who make the observations and the techniques that they are allowed to use. This is one of the big differences between 10 years ago and today. Many more methods have come to our disposition. The problem is as old as the cell problem really is, but it changes because of the different approaches that can be made in the course of time.

We are especially covering the questions of compatibility or incompatibility of the final mechanisms of cell differentiation and the comparison of *in vivo* and *in vitro* procedures. We will hardly touch the processes covering the early stages where still truly molten cells switch off, in some way, many of their potentiations and end up with one or two possibilities of expression.

Certainly many more questions can and should be asked and answered, such as what our messengers for differentiated function produce during subsequent phases of cell division and, especially, what happens in this respect during the S phase while DNA duplication occurs.

Growth Rate and Differentiated Function of Cells^{1, 2}

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SUMMARY

Observations on muscle tissue from chick embryos are taken as a point of departure for discussion of the relation of cell proliferation and cell differentiation during embryonic development. In the leg musculature the proportion of cell nuclei in mitosis or in DNA synthesis dropped rapidly after the 7th day of incubation and declined more gradually from the 11th day of incubation until the 9th day after hatching. During this developmental period an increase in the duration of the intermitotic phase of the replicating cells was indi-

cated. In contrast to the sharp decrease in proliferative activity during the early portion of the investigated period of development, the concentration of myosin, one of the main cell-specific products of differentiation, increased only slowly up to the 16th day and reached a maximal rate of accumulation only on the 18th day of incubation. The relation of these findings to past work in this field and to possible future approaches is discussed.—*Nat Cancer Inst Monogr* 26: 303–326, 1967.

TO BEGIN a discussion of the relation of proliferative growth and differentiation I will recall the roots of experimental work from which the contemporary studies in this area evolved, and summarize some of the main advances made since the first Decennial Tissue Culture Conference (DTC), including recent work in our laboratory on quantitative aspects of the transition from proliferation to differentiation in muscle of the intact chick embryo. Finally, I will attempt to define several questions arising from the reviewed material as a basis of further discussion and, possibly, of further work.

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The studies of differentiation and proliferation in culture have separate origins. Originally, tissue culture started as a method for maintenance of embryonic tissues *in vitro* (1, 2), and the chief purpose of cell culture was not the achievement of high rates of proliferative growth. This situation was reversed with the discovery of the proliferation-stimulating property of embryo extract (3) which promised possibilities for quantitation of cell growth, for establishment of so-called pure cell strains, and perhaps for new insights into the nature of neoplasia. This new approach spread rapidly to other laboratories while the tradition of organ culture as a tool for the study of embryonic differentiation found shelter only at few places, foremost at the Strangeway Laboratories. During this period of compartmentalization of cell culture and organ culture, questions about the relationship of proliferation and differentiation were not intensively investigated or widely discussed. A noteworthy exception is Strangeway's book, "Tissue Culture in Relation to Growth and Differentiation" (4).

A beginning convergence of *in vitro* proliferation and differentiation studies can be seen in the work of Fischer and Parker (5), which suggests that, even after long periods of rapid proliferation, tissues maintain characteristic differences. Subsequent experiments such as those by Doljansky (6) were thought to indicate that proliferation and at least the full expression of differentiation are mutually exclusive processes and, depending on culture conditions, one trait or the other could become predominant (for review *see also* 7, 8). The idea of an incompatibility of proliferation and differentiation has remained current among embryologists and cytologists (9-13). Recent rigorous reexaminations of the assumptions and results of the work of this period can be found in reviews by Grobstein (14) and Konigsberg (15).

Three main questions emerge from the work on proliferation and differentiation carried out up to the preceding DTC:

1) Is there complete incompatibility of proliferation and differentiation in the sense that cells still in some phase of proliferation do not synthesize any cell-specific products at all?

2) In what sense does the transition from proliferation to differentiation lead to loss of proliferative capacity and, conversely, the transition from differentiation to proliferation lead to an equal loss of capacity to differentiate?

3) If redifferentiation occurs after prolonged periods of proliferation, can the properties of the redifferentiated cells differ qualitatively from the properties of the cells from which the proliferating cell was derived originally? This possibility has been generally discounted, with the exception of Wolffian regeneration, but just at the time of the first DTC this question was reopened by Trinkaus (16) and Ephrussi (17).

Work done since the first Decennial Tissue Culture Conference, both *in vitro* and *in vivo*, has brought some clarification of these questions. Demonstration of complete dissociation of proliferation and differentia-

tion was obtained by investigations of developing muscle. Holtzer, Marshall, and Fink (18) observed that somite cells binding fluorescein-labeled antimyosin to myosin do not possess mitotic figures. Subsequently, Stockdale and Holtzer (19) demonstrated that cultured muscle cells incorporating thymidine-³H, indicating DNA synthesis, do not bind fluorescent myosin antibody, while cells in which DNA synthesis has ceased contain some material, presumably myosin, which do bind myosin antibody.

Supporting evidence that muscle fibers lack DNA synthesis has been added by several investigators using microspectrophotometry of Feulgen-stained nuclei. Lash, Holtzer, and Swift (20) found only 2 N amounts of DNA in nuclei of multinucleate myotubes, but 2 N-4 N amounts were measured in the mononucleate cells outside the myotubes of regenerating muscle. These results were corroborated by spectrophotometric measurements of nuclei of developing muscle cultured *in vitro* (21-24). Interestingly these experiments confirm observations on muscle made some time ago. Lewis and Lewis (25) reported absence of mitotic figures from the multinucleate myotubes formed in outgrowths of muscle explants.

With the use of markers for two phases of the cell replication cycle, mitosis, and labeling of DNA, the method of "labeled mitosis" of Quastler and Sherman (26) led to a sharper definition of the replicative state of cells, adding evidence for the separation of cell replication and differentiation. Cells from the zone of rapid proliferation in the crypts of the mouse small intestine were followed through their migration up the villi, where they became nondividing specialized cells that eventually sloughed into the lumen of the intestine. Stem cells themselves had a very slow dividing cycle compared to cells that migrate up the villi and proliferate rapidly. However, cell replication ceased before full histological differentiation occurred (27). Young (28) used the same technique to demonstrate that osteoblasts and osteoclasts are specialized cells in bone-forming tissues that had ceased to synthesize DNA.

Analyses by several other investigators indicate a less clear-cut separation of proliferation and differentiation. Wessells (29) studied the appearance of the postmitotic cell population in pancreas which produces zymogen granules as specialized products. Division figures were found in cells containing small amounts of zymogen, although it was unclear whether zymogen synthesis began only after DNA replication was completed. However, low levels of amylase activity were detected in proliferating mesenchyme cells of the pancreatic primordium 4 days before prozymogen granules appeared (30). This suggests an overlap of cell proliferation and the formation of low levels of cell-specific proteins. Nossal and Makela (31), studying maturing plasma cells, demonstrated that thymidine incorporation and, presumably, mitosis cease before maximum production of antibody. They suggest, however, that a very small portion of the early

plasma cell population can synthesize DNA after production of specific antibodies has begun.

Similarly, lens development studies indicated an incomplete separation of proliferation and differentiation. Papaconstantinou (32) examined by chromatographic methods the soluble proteins in calf lens and found α - and β -crystallins in both proliferating epithelial cells and differentiating fibers, but γ -crystallins were present in only the differentiating fibers. It was concluded that the γ -crystallins are proteins specifically related to the process of lens fiber formation. Yamada and associates (33) detected α - and β -crystallins immunologically in the germinal epithelium of the regenerating lens. These epithelial cells continue to divide as they move toward the periphery of the lens where they differentiate into fiber cells, but γ -crystallins are produced only after the cells begin to form protein fibers. Autoradiographs showed that DNA synthesis ceases before the cells start fiber differentiation. When the immunofluorescent reagent is used on thymidine-labeled tissue, the final phase of DNA synthesis is separated from γ -crystallin formation by a time span of 1.5 days. Noteworthy is the maintenance of low levels of production of cell-specific proteins in cell lines derived from tumor tissues (34-36). A broad documentation of the expression of cell-specific properties during cell culture is given by Harris (37) and Eagle (38).

Formation of cell-specific proteins at very early stages of development is reported for lens proteins (39-46), and hemoglobin (47-49). Whether this means that under these conditions differentiation and proliferation occur simultaneously in many cells, or that full differentiation is restricted to few cells which terminate their proliferation at a very early stage of development has to be reexamined.

Studies by Ebner *et al.* (50) on proliferating cultures of the mammary gland showed that the levels of two different cell-specific enzymes decline at different rates. In nonproliferating cultures of the thyroid, Hilfer (51) observed that the elaboration of thyroid hormone declines even though proliferation does not take place.

This wide range in possible relations of proliferation and differentiation, summarized by Harris (37, pp 153-154) and supplemented by a report on the "Retention of Functional Differentiation in Cultured Cells" (52) makes one pause in reconsidering these cell processes. Together, these results strongly suggest that the assumption of a complete and general mutual exclusion of DNA synthesis and cell replication on the one hand and production of cell-specific proteins on the other cannot be accepted without reservation and that a certain degree of overlap may exist.

As a further step in exploring the relation of proliferation and differentiation, it could be shown that the loss of cell-specific properties that occurs during cell proliferation can be reversed on cessation of proliferative activity. Konigsberg (53) demonstrated that fibrils and cross-striation which disappear during cloning of muscle cells are again formed when

cells fuse into myotubes in colonies after about 1 week of culture. Similarly, reformation of cell-specific products can be seen in cultures of cartilage cells after a period of proliferation (54). Factors which control such "redifferentiation" in cultures of cartilage and iris cells have been recently investigated (55-57).

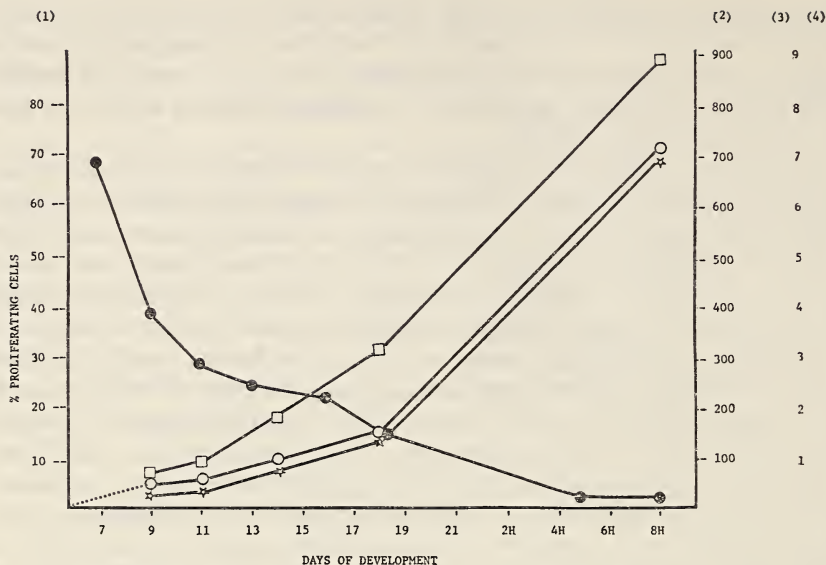
Work done in the last decade has contributed significantly to the elucidation of qualitative aspects of the relationship of proliferation and differentiation, mainly in *in vitro* model systems. Our laboratory has sought a quantitative definition of this relationship, with muscle being mainly used in our investigations. Initially it seemed desirable to investigate muscle development in the intact embryo, since there are no techniques that permit observation *in vitro* of the entire transformation from a rapidly proliferating to a highly differentiated tissue. Hence, analysis of development in the embryo remains the only way to define the full sequence of events which effect this transition. Observation of the transition from proliferation to full differentiation in the embryo provides data that may serve as a basis for comparison with the results of corresponding analyses of similar tissues maintained in culture. Progress of this work has depended on adaptation of methods for detailed analysis of changes in cell population of the developing muscle, and for quantitative recovery in a highly purified state of the cell constituents used in tracer studies and immunological assays.

Our work was done with chick leg muscle from the 7th day of incubation until the 8th day after hatching. On the 7th day of development the myogenic cells showed a higher mitotic activity than the surrounding mesenchyme cells (58). This indicates an increase in proliferative activity during the initial phase of myogenesis. The relationship of the decline in proliferation to the progress of differentiation, as measured by myosin increase, is apparent from the juxtaposition of the curves for the proliferation index and myosin levels in developing leg muscle (text-fig. 1).

The data for the proliferation index were obtained (59) by a procedure based on a method of Quastler and Sherman (26) and elaborated by Cairnie *et al.* (27). The quantities of myosin were determined for chromatographically homogeneous preparations obtained by a method developed in our laboratory (60). On the 7th day of development, 1-2 days after formation of myogenic tissue, about 30% of the cells had ceased to proliferate.³

During the following 4 days, the number of nonproliferating cells increased rapidly, and by the 11th day 70% of the cells had ceased to proliferate. The rapid drop in the number proliferating cells was followed by a more gradual decline to very low values during the posthatching period.

³ During the transition from proliferation to differentiation the mononucleate myoblast cells fuse into multinucleate myotubes that differentiate into muscle fibers. The data on proliferation reported here were obtained by observation of cell nuclei in either mononucleate cells or the nucleocytoplasmic units after fusion. The term "cell" is thus used for convenience only and refers either to mononucleate cell or nucleocytoplasmic unit in the muscle fiber.



TEXT-FIGURE 1.—Percentage of proliferating cells and myosin content in chick leg muscle during development from 7 days' incubation to 8 days after hatching (H).

1. ●—● Number of proliferating cells in percent of total cell population. The data are taken from Marchok and Herrmann (59).
2. □—□ Microgram of myosin/g tissue (fresh weight). The data are taken from Baril and Herrmann (61).
3. ○—○ Myosin/DNA of nonproliferating cells.
4. ☆—☆ Myosin/DNA of total cell population.

If differentiation, defined by myosin accumulation, were to follow directly upon cessation of cell replication, the most extensive rise of myosin concentration in the developing leg muscle would be expected also during the 9–14 day period of development. But the decline of proliferation and the increase in amount of myosin did not follow this expectation, and their relationship is more complex and in certain respects still unclear. It seems definite that myosin accumulates at the highest rate almost 1 week after the most rapid decline in the number of proliferating cells (61). This could mean that rapid formation of other cell-specific products, perhaps actin or tropomyosin (62), immediately follows cessation of proliferation with other specific products, increasing later in a staggered fashion (*e.g.*, myosin shows a 1-week lag). An alternative interpretation is that rapid formation of myosin and, perhaps, of other cell-specific proteins is delayed until another phase of cellular maturation has occurred after cessation of cell proliferation. On the 11th day only 13% of all nuclei were found in myotubes or myofibers (59) although proliferation had ended in about 70% of all nuclei. Thus, synthesis of material required for myotube forma-

tion may be one of the processes which immediately follow cessation of proliferation and precede rapid synthesis of specific muscle proteins.

More detailed examination of the curves for decline of proliferation and increase in myosin concentration may reveal a biphasic course for both parameters. In addition to extensive and rapid increase of myosin after the 16th day, the possibility of an earlier, shorter, and less rapid absolute increase is indicated. Since condensation of myogenic tissue occurs at the end of the 5th or the beginning of the 6th day, the myosin content would be at zero level at this point of development. In this case the increase in myosin from a zero value on the beginning of the 6th day to a value of 0.33 mg/mg DNA or 0.67 mg/gm fresh weight on the 9th day would be more rapid than the very slow increase between the 9th and 14th day. Hence, the rapid and extensive increase after the 16th day of development described earlier, would then represent a second phase in the acceleration of myosin synthesis. The period of most rapid decline of proliferation, from the 7th–11th day of development, was followed by a period in which proliferation decreased very slowly, but a second more rapid decline is perhaps indicated at about the 18th day.

RNA precursors are incorporated into RNA of muscle nuclei more rapidly during the same two periods (63), but a direct relation between increased synthesis of myosin and RNA has not been demonstrated. Also, the interpretation of the low myosin level found for the 9th day is uncertain. At such low myosin levels a significant part of the myosin fraction may be contributed by contractile elements of the vascular system or by proteins involved in general cell motility (64). This significantly lower value for "true" myosin for the 9th day would give a continuously accelerating myosin increase.

The basic observation of a transition from full proliferation to full differentiation leads to several questions. Some of these can be answered tentatively. Others may lead to discussion or further work.

What is the role of the proliferation preceding differentiation?

In muscle development, condensation of myogenic tissue is accompanied by an increase in proliferation rate above the level of the primitive mesenchyme from which the myogenic tissue is derived. There is a similar increase in proliferative activity during the migration of intestinal epithelium and its transformation from a stem cell to the fully differentiated intestinal epithelium (26). Is this spurt in proliferation solely to supply the number of cells required for the attainment of the final size of the respective organs or for replacement of lost cells? Or do stem cells or other precursor cells such as primitive mesenchyme have a low but definite degree of specialization, *e.g.*, fiber formation, which must be lost during a proliferative phase to permit a more divergent specialization, *e.g.*, cartilage or muscle formation. Are separate stimuli, then, required to initiate pro-

liferation, the condensation following proliferation, and subsequent specialization; or are these processes triggered by the same condition? Results pertaining to these questions were recently obtained by Stockdale and Topper (65) who found that explants of mammary glands respond to prolactin and hydrocortisone with an increased production of casein only after a preceding stimulation of proliferative activity by insulin.

Is there a definite number of cell divisions before proliferation ceases?

The continuing decline of proliferation over a long period of muscle development can be accounted for in two ways. Assuming that proliferation ceases after the same number of divisions in each cell, the duration of the replication cycle must increase in the cells the later in development their proliferation comes to a standstill. Conversely, the duration of the replication cycle could be maintained at the same value and the number of divisions would be higher for the cells that cease proliferating later. Both conditions could contribute synergistically.

The duration of the phases of the replication cycle were determined in the developing muscles that we studied (62) by the procedure of Quastler and Sherman (26) and the values are given in table 1. These figures were used to approximate the duration and number of replications for cells terminating proliferation at different days during the investigated period of muscle development. In these calculations it was assumed that the measured duration for the replication cycle of 10 hours and 16 hours for the 9th and 16th day of development, respectively, indicates an increase of about 1 hour/day of development (rounded off from 0.86 hours as calculated in table 2). On this basis, cells that cease proliferating at points of development from the 7-day embryo to 8 days after hatching would show an increase in the total number of replications from 6–20, and in the duration of replication cycles from 8–29 hours (table 2). In this instance it seems most likely that both an increase in replication time and in the number of replications lead to the wide spread of termination of proliferation.

TABLE 1.—Duration of phases of the replication cycle in proliferating cells of the developing leg muscle of the chick*

Phase	Duration (hours)	
	9-Day embryo	16-Day embryo
DNA synthesis (S).....	5.85	5.85
Postsynthetic + mitotic ($G_2 + M$).....	1.75	1.75
Postmitotic — presynthetic (G_1).....	3.90	8.90
Total cycle ($S + G_2 + M + G_1$).....	10.50	16.50

*The data are taken from Marchok & Herrmann (59).

TABLE 2.—Proposed hypothetical change in duration of replication cycle and number of replications for cells terminating proliferation at the indicated days of development

Day of development	Time elapsed from beginning of myogenesis on the 5th day (hours)	Duration of replication cycle (hours)	Number of replications
5.....	—	—	—
7.....	48	8	6.4
9.....	96	10*	11.3
16.....	264	16*	24.3
18.....	312	18	27.0
29†.....	576	29	38.2

*Figures obtained (59) by measurements according to Quastler and Sherman (26). The other values for the duration of the replication cycle were calculated on the basis of a 1-hour increase per day of development. The 1-hour value is the rounded-off figure for the average daily increase calculated from the difference in the duration of total replication cycles for the 9th day (10.5 hr) and 16th day (16.5 hr) (see table 1). With a difference of 6 hours for the duration of the replication cycle for the elapsing 7 days, a daily increase of 0.86 hours is obtained.

†Hatched 8 days.

Are there discrete levels of differentiation?

According to the model of Jacob and Monod, differentiation results from gene derepression followed by transcription with production of cell-specific proteins. In this sense derepression would be an all-or-none process, and production of cell-specific proteins should take place immediately after initiation at a maximal rate and should continue at that rate until a maximal level of the respective proteins found in the mature cell is reached. Hence we may ask why the initial production of cell-specific proteins such as myosin apparently starts slowly and increases only gradually, or why in some instances production of cell-specific proteins remains for some time at the same low level.

Since developing muscle tissue is a nonuniform cell population, a gradual increase in the rate of protein accumulation may mean that some cells are derepressed earlier and others later, but that derepression always leads to production of the respective proteins at a maximal rate. Similarly, where a low level of a certain protein is maintained during some period of development, it could be that only a small fraction of all cells are derepressed but that synthesis in these cells occurs at a maximal rate.

Alternatively, the change from a low to a high rate of synthesis could occur within each cell of a large segment of the cell population. In this case any one of the numerous regulatory mechanisms affecting transcription or translation of the genetic code could account for this change. Extensive work is being done along these lines.

Additional minor considerations include the indication of a large increase in the duration of the G_1 phase of the replication cycle. This phase, comprising the period after conclusion of mitosis and before onset of DNA

synthesis, increases in developing muscle from 3.9 hours on the 9th day to 8.9 hours on the 16th day of development. Probably it reaches 15 to 20 hours during the posthatching period. It would be remarkable if, during G_1 phases of such long duration, synthesis of cell-specific proteins did not occur but would be initiated only after complete cessation of proliferation.

What is the relation between the rate of total protein synthesis in a cell and formation of cell-specific proteins?

This question emphasizes the lack of data on elementary kinetics of protein synthesis during the transition from proliferation to differentiation. Does the rate of synthesis of cell-specific proteins begin to increase only after the rate of total protein synthesis required for proliferation has reached a critical, low value? Are there limiting factors needed for transcription and translation which control this relationship? In muscle, the increase in synthesis of myosin occurs (61) after proliferation (59) and increase in dry matter (66) has slowed down. But, in the developing cornea (67) collagen synthesis increases while total protein synthesis does not decline. From these preliminary data it appears that total protein synthesis and synthesis of cell-specific proteins can assume different relations, and that the former does not necessarily control the latter.

To what extent are in vitro maintenance and in vivo development comparable?

Similarities in morphogenesis, mainly of skeletal structures, obtained on development of bone rudiments *in vitro* or in the embryo, have been recognized since the early experiments at the Strangeways Laboratories. Several instances of a maintenance of normal histology and even of characteristic pathological changes have been observed in organ cultures since that time and are reported in two symposia (68, 69). A quantitative correspondence between explants and the tissue *in situ* is indicated by the assays for glutamine synthetase in organ cultures of the retina (70).

However, in cultures of retina cells reaggregated after a period of rapid proliferation, the synthetase level remains lower than in the organ cultures or in the tissue in the embryo. Steps toward a better understanding of conditions which may promote or maintain high levels of cell-specific proteins in cultures may be represented by work mentioned before (55-57, 65) and by the experiments discussed earlier by Amos (71). Also, the role of hormone-like substances which stimulate terminal steps in the differentiation (72) of certain tissues must be explored from this point of view.

These efforts converge toward one central issue: the experimental control of proliferation and differentiation in cell and organ culture. The change from differentiation to proliferation as well as from proliferation to differentiation requires DNA-dependent RNA synthesis. In this sense, interconversion of proliferation and differentiation is similar to the induction of a specific differentiation itself. However, although production of proteins needed for proliferation on the one hand and for specialization, on the other, can so readily be reversed, attempts to change production of

protein from one type of specialization to another have so far failed. We hope that the reasons for this fundamental difference will soon be elucidated.

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DISCUSSION

Dr. Konigsberg: I would like to take advantage of two fortuitous circumstances. One, that the organizing committee chose as a general unifying theme for this Conference the relationship of the cultured cell to its *in vivo* progenitor; the other, that Dr. Herrmann, although he has dealt primarily with studies of myogenesis *in vivo*, has raised certain provocative questions relative to the relationship of cultured muscle cells to their *in vivo* progenitors. I would like, therefore, to describe myogenesis in a culture system under conditions of single-cell cloning. Since this culture technique probably represents the most radical departure from the situation *in vivo*, it might be profitable to compare the developmental sequence in muscle clones to muscle differentiation *in vivo*.

The progenitor cells I shall discuss are embryonic leg-muscle cells of the chick embryo isolated from 11- to 12-day muscle. Such cells are suspended by trypsinization and cloned by essentially the techniques developed in Puck's laboratory.

Figure 1 illustrates a culture initiated with 200 cells. The culture was incubated for 2 weeks and during this time discrete colonies formed. These colonies fall into two distinctly different morphological classes. There are colonies which are generally spherical and composed of cells which we can classify only as "fibroblastic in appearance." The other type of colony, having an irregular outline, appears macroscopically to be composed of long fibers. These are, in fact, colonies containing differentiated muscle elements which, being elongate, multinucleated cells, give the colony its fibrous appearance. We can demonstrate the presence in these multinucleate cells of the pattern of cross-striation typical of skeletal muscle fibers.

Not only can we identify the macroscopic muscle colony but we can identify, as well, the progenitor cell which will produce the muscle colony. Single myoblasts, by gross cell shape alone, can be distinguished from fibroblastic cells. Myoblasts, in culture, possess a characteristic bipolar shape quite similar to the appearance of cells in myogenic areas *in vivo*.

Figure 2 contrasts the appearance of an early muscle colony with that of a colony of fibroblastic cells on the 4th day of culture. The spindle shape of the myoblast is dramatically evident in these photomicrographs due to the amplification afforded by the degree of cell replication that has occurred. Incidentally, Warren and Margaret Lewis in their pioneering studies observed such spindle-shaped cells in cultured muscle explants and suggested that these were the myogenic elements.

Using this criterion of cell shape, we were able to select and follow single myoblasts through successive stages in the formation of a muscle colony. The first 4-5 days in culture marks a period of fairly rapid proliferation. Counts of cells per colony during this period indicated a generation time of 12-18 hours. This short generation time surprised us and we were inclined to regard it as a culture artifact. It is gratifying, however, that Dr. Herrmann has found generation times of the same order in muscle tissue *in vivo*.

After this initial period of cell replication we see, on the 5th and 6th day, the initiation of the formation of the first myotubes. Myotube formation occurs by a process of cell fusion, and nuclei once incorporated into a myotube no longer replicate. Therefore increase in size of the muscle colony occurs by division of the mononucleated cells scattered throughout the colony. The network of myotubes, on the other hand, increases in number and length by recruitment from this pool of mononucleated cells. Thus, after myotube formation has been initiated, we can see, at all subsequent stages, both mononucleated cells and myotubes within the same colony. (On rare occasions, all the cells of an earlier muscle colony will fuse during a short time interval. Such colonies, however, never enlarge and remain microscopic.) Generally we do not see the process that was illustrated in the first figure Dr. Herrmann showed us. His figure shows the progressive formation of muscle fibers and the diminution of the number of cells in the spaces between muscle fibers with increased embryonic age *in vivo*. This difference between clonal development and muscle differentiation *in vivo* may be a consequence of the peculiar geometry of muscle cells growing as a two-dimensional colony from a single cellular progenitor. Such colonies never attain the cell density achieved *in vivo*.

The most significant difference, however, between myogenesis in clones and *in vivo* is that muscle cells within a pure clonal colony differentiate in the absence of fibroblasts with which, *in vivo*, these cells would have been intimately associated. One might assume that the fact that differentiation occurs under these conditions would indicate that myogenesis is not dependent on any contribution from the fibroblast. This assumption is untenable because to achieve cloning of individual muscle cells, we have had to resort to the use of conditioned medium, that is, medium conditioned by large populations of freshly isolated cells from skeletal muscle. This medium, then, is re-utilized to establish the clones. This in itself should indicate that the muscle colony is not completely independent of any contribution from other cells.

More recently, Dr. Steven Hauschka and I have obtained evidence that strongly suggests that the active component of the conditioned medium is, in fact, collagen, a biosynthetic product of the fibroblast. Without going into detail, the following considerations led us to this premise:

We found that conditioned medium apparently affected the surface of the petri plate primarily, not the cells *per se*. If we pretreated petri plates with conditioned medium for different periods, aspirated off the medium, rinsed the plates, and then used these same petri plates to culture cells in unconditioned medium, they behaved qualitatively and quantitatively just as they would have if they had been grown continuously in conditioned medium.

We also had some indications that the cells responsible for the conditioning were the fibroblasts.

In speculating about what products of the metabolism of fibroblasts might represent the material deposited on the petri plates by the conditioned medium we quite naturally considered collagen. We were encouraged to consider this possibility more seriously when some preliminary electron-microscopic studies indicated the synthesis of at least collagen-like fibers in our conditioning farms.

More recently, Dr. Hauschka, using tracer techniques, obtained evidence that in the conditioned medium a newly synthesized protein is present. This protein, having the

solubility properties of collagen, contains large amounts of hydroxyproline in the appropriate range for collagen and, when petri plates are treated with labeled conditioned medium, this protein is adsorbed to the surface of the petri plate.

As a rapid test of the hypothesis that collagen was, indeed, the active component of the conditioned medium we cultivated our cells on a substratum of precipitated collagen; this was done much in the manner described by Ehrmann and Gey, and by Bornstein. In the presence of unconditioned medium, by using a substratum of collagen, we found that we could dispense with the use of conditioned medium.

Figure 3 illustrates the results of such an experiment. Of the three petri plates shown, A and B are controls; that is, cells cultured in unconditioned medium (A) and in conditioned medium (B). In unconditioned medium fibroblast-like colonies are present but none of the large fiber-containing colonies of muscle cells. The petri plate shown in C of this figure is a culture of cells grown in unconditioned medium but on a collagen substratum.

The same medium was used in both the A and C groups; the difference between them is due to the presence of the collagen substratum. So the use of the collagen substrate does substitute for the use of conditioned medium.

There are many things we don't know about the problem: For example, which component processes in the development of the muscle colony are affected by collagen? Is this dependency of myogenic elements on fibroblasts a property of cells in culture exclusively or does it have some real significance in the *in vivo* situation. To get at the latter question, it is unfortunate that we can't use the elegant techniques those people working on epithelial-mesenchymal interactions can apply to strip off one cell type from the other. Since in muscle tissue the myoblast and fibroblast are intimately intermixed, I suppose the lesson is not to work on mesenchymal-mesenchymal interactions because they are more difficult to get at and more difficult to pronounce.

Dr. Harary: I would like to discuss the general theme of this session, as reviewed by Dr. Herrmann and Dr. DeMars, concerning the relationship between proliferation and differentiation and gene expression. I shall orient my remarks toward a possible redefinition of the problem on a more nearly molecular basis because I think it is possible to view this problem divorced from the cell cycle and cell division. One may be able to investigate the relation of proliferation and differentiation by studying the control mechanisms involved in routing the cell toward special synthesis, such as the synthesis of special proteins, or in routing the cell toward the duplication of the general proteins necessary for cell multiplication.

I shall illustrate these points by some work we have done in our laboratory in the last several years on cultured rat heart cells. This is a system of monolayer of rat heart cells derived from a neonatal rat. These heart cells can continue to beat for up to 6 weeks. But in most of the experiments described, the conditions were set up so that the cells stopped beating after 10-14 days.

One of the first things we discovered about these cells was that during this 2-week period they dedifferentiate. This term is used mainly to designate a loss of function and a change in enzymes associated with this loss of function, with no implication that dedifferentiation means a return to a primitive state. As shown in table 1, these cells lose the ability to oxidize fatty acids very quickly. They don't lose the ability to metabolize glucose. Glycolysis stays fairly constant. There is no change, for instance, in the enzyme hexokinase. There is a drop in respiration and oxygen uptake. Two enzymes, isocitrate dehydrogenase and malic dehydrogenase, decrease to about 10% of the original value in these cells. Glucose-6-phosphate dehydrogenase increases about tenfold. More significantly, there is a decrease in myosin, which we measure as calcium adenosinetriphosphatase and in creatine kinase—enzymes, which are more closely representative of the function of the heart cells.

TABLE 1.—Changes in cultured heart cells*

	Days in culture	Activity
Isocitric dehydrogenase.....	1	1, 380
	8	150
Malic dehydrogenase.....	1	15, 000
	8	850
Calcium adenosinetriphosphatase †.....	0	0. 87
	11	0. 04
Creatine kinase‡.....	0	48
	3	5
Hexokinase.....	1	0. 23
	8	0. 25
Glucose-6-phosphate dehydrogenase.....	1	58. 5
	6	197
Pyruvate oxidation.....	1	5. 3
O ₂ uptake μ liter/hr/mg dry wt.....	8	2. 7
Palmitate oxidation.....	1	11. 4
O ₂ uptake μ liter/hr/mg dry wt.....	8	0
Glucose oxidation §.....	1	+24%
% change of endogenous.....	6	-19%
Respiratory quotient.....	1	0. 81
CO ₂ produced.....	14	0. 96
O ₂ absorbed.....		

*Enzyme concentrations were measured in extracts prepared as described in Kuramitsu, H., and Harary, I.: *Biochim Biophys Acta* 86: 65, 1964. Pyruvate and palmitate oxidation were measured in whole cells and are expressed as the increase O₂ uptake caused by the addition under conditions quoted in the reference.

† μ moles/pi/5 min/mg protein.

‡ μ moles/pi/min/mg protein $\times 10^3$.

§Change in O₂ uptake μ liter/hr/mg dry wt with 0.02 M glucose.

So essentially, these cells, which oxidize fatty acids and glucose, become primarily glycolytic cells and lose some of the enzymes that are specifically related to heart function. These cells grow, with an increase in the number of cells and in protein. There is also an increase in the total amount of calcium adenosinetriphosphatase initially, but this increase is smaller than the increase in the protein, leading to a decrease in the specific activity. Eventually, of course, even the total amount comes down close to zero. And the question one asks is whether the reason for this shift in metabolism and so-called dedifferentiation is a result of the onset of proliferation and cell division.

There is a very simple experiment to test this. You can add colchicine to the cells to stop division. If you look at the same parameters, you will find that the calcium adenosinetriphosphatase and the malic dehydrogenase do not decrease in these cells. Also, in the colchicine cells, glucose-6-phosphate dehydrogenase does not increase. The total activity and the specific activity in complete growth medium increases markedly. When colchicine is added, there is no change in the total activity and some change—actually a decrease—in the specific activity. But this is not too significant. It would seem that, from this point of view, if you inhibit cell division, you inhibit dedifferentiation and maintain the cells in culture. The cells can be maintained for quite a long time with colchicine.

However, it can be shown that dedifferentiation may occur without cell division. One can, for instance, inhibit cell division by other means and show that there can be dedifferentiation in the system. We incubated these heart cells on a medium containing lipid-free albumin and no serum. These cells did not grow or divide. And yet

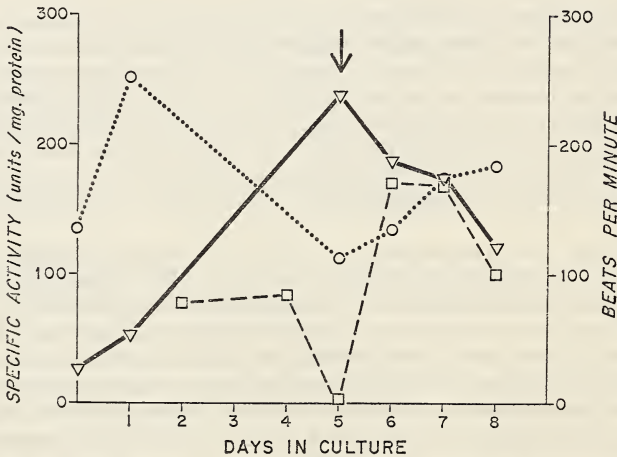
TABLE 2.—Effect of Colchamide on glucose-6-phosphate dehydrogenase

Days in culture	Complete growth medium		Complete growth medium + Colchamide*	
	Specific activity	Total activity	Specific activity	Total activity
1.....	17.2	2		
3.....	44	7.6	40	3.0
7.....	104	40	12	1.0
10.....	96	64	27.2	3.6
15.....	172	240	16	1.6

*Colchamide was added on day 1 (final concentration: 1 μ g/ml).

they underwent the same dedifferentiation process as the cells that do grow and divide. Text-figure 1 shows the changes in enzymes in nongrowing cells. There was a decrease in the malic dehydrogenase and an increase in glucose-6-phosphate dehydrogenase up to the 5th day and a cessation of beating when serum lipids were added (*at arrow*). There was a return of the glucose-6-phosphate dehydrogenase toward the original value of the glucose-6-phosphate dehydrogenase and also a return of the malic dehydrogenase toward the original value of malic dehydrogenase.

It is interesting to ask, then, whether this change has anything to do with cell division or whether it is a result of some early decision on the part of the cell on which way to go and whether lipids may have some relationship to this process. We added colchicine to the cells which were not going to divide in any case, and the enzymes of the cells were also maintained.



TEXT-FIGURE 1.—Enzyme changes in lipid-deficient and lipid-supplemented media. The cells were cultured in growth medium with purified lipid-free albumin. Malic dehydrogenase $\bigcirc$ — $\bigcirc$ (specific activity $\times 10^{-2}$) and glucose-6-phosphate dehydrogenase $\triangle$ — $\triangle$ were determined at the outset and on the 2d and 5th day. On the 5th day the beating $\square$ — $\square$ stopped. At this time, indicated by *arrow*, the medium was changed to one supplemented with serum lipids at a concentration equivalent to that used in the complete growth medium which contains 20% serum.

This, of course, is not consistent with our understanding of what colchicine does. Colchicine has been thought to cause metaphase arrest. However, it is possible that colchicine has an effect on an early event in the cell, an event related to the preparation for cell division. Colchicine may affect the shunting of metabolism or the conditions in the cell concerned with specialized function. In support of this concept of the mechanism of colchicine is an experiment we recently did on inhibition of cell division with irradiation. If cells are irradiated with 2000 r, they do very poorly.

After irradiation, death was prevented and the cells were maintained very well in culture with colchicine. In other words, if you prevent the cells from reaching the point of using the damaged DNA for replication, it is possible that you can maintain the cells in the differentiated state.

My only point is that it is possible to consider these questions of proliferation and specific function in a different light. The problem may be redefined in an experimental way, which may place it on a more molecular basis.

Dr. Holtzer: Since much of the material I will discuss has been published,¹⁻⁴ my contribution will be confined to a few brief and, I hope, constructively provocative observations.

One of the central problems in cell differentiation is: How many different kinds of molecules can a cell synthesize concurrently? For example, I should not be surprised if it were demonstrated that in the liver cell the enzymes required for oxidative phosphorylation are synthesized at the same time that the cell makes albumin. However, I do not believe it probable that a liver cell can concurrently synthesize albumin and myosin or hemoglobin. As I visualize it, the normal ongoing cytoplasmic-nuclear dialogue in a liver cell or pancreatic cell, or even a zygote, or blastula, or gastrula cell is such as to preclude the functioning of the machinery required for synthesizing myosin or hemoglobin.

Similarly, the old problem of proliferation versus differentiation may be rephrased in these terms—whether the involvement of a cell in making one kind of molecule effectively excludes it from making certain other kinds of molecules. Specifically, it may be asked whether a cell can make all of its various molecules during any stage of its life cycle. Is transcription or translation paced or influenced by the stage of mitosis in which the cell is? By definition, a cell in G₁, or G₂, or M does not make DNA. Given the depression of total protein synthesis in most cells during M, it is likely that even the ubiquitous glycolytic enzymes are not synthesized at that time. There is then precedent for framing the question: Does the commitment of a tissue cell to make molecules appropriate for one period during the mitotic cycle exclude the formation of molecules appropriate to another phase of the mitotic cycle, much as a commitment of a cell to albumin synthesis precludes its translating for myosin?

Against this background I should like to review some experimental data which points to the fact that normally a myogenic cell in the mitotic cycle does not synthesize myosin. To analyze what “a cell” and not “a population of cells” may be doing, it is obviously not permissible to take a “piece” of embryonic muscle and correlate its content of DNA with its content of myosin. A “piece” of muscle consists of a heterogeneous population of cells, over 60% of which are not even myogenic, *e.g.*, connective tissue cells, etc. Consequently, the determination of whether a single cell can make DNA and myosin simultaneously requires techniques which permit the inspection of single cells. Radioautography, with the use of ³H-thymidine, can be used to identify single cells making DNA and fluorescein-labeled antibodies against

¹ HOLTZER, H.: *In* Molecular and Cellular Synthesis, 19th Growth Symposium. New York, The Ronald Press, 1961.

² HOLTZER, H., ABBOTT, J., and CAVANAUGH, M.: *Exp Cell Res* 16: 595, 1959.

³ OKAZAKI, K., and HOLTZER, H.: *J Histochem Cytochem* 13: 726, 1965.

⁴ OKAZAKI, K., and HOLTZER, H.: *Proc Nat Acad Sci USA* 56: 1484, 1966.

myosin, and the polarizing microscope can be used to ascertain whether single cells have made myosin.

The results of these experiments are reasonably straightforward. Cardiac (Holtzer, Abbott, and Cavanaugh, 1959) or skeletal myogenic cells with mitotic figures did not bind antibodies to myosin. Myogenic cells incorporating ^3H -thymidine did not bind the antibodies. Conversely, myogenic cells that bound the antibodies did not display mitotic figures nor did they incorporate the DNA precursor.

Fusion is another activity associated with myogenesis which is paced by the mitotic cycle. By pulsing cells with ^3H -thymidine and then determining the time required for labeled cells to fuse, it was found that fusion did not occur in S, G₂, or M. Cells fuse only after a minimal period of 3–5 hours in G₁.

In another series of experiments, replicating myogenic cells were exposed to ^3H -thymidine for 2, 4, 6, 10, and 15 hours. The cells were then treated with labeled anti-myosin and radioautographed. There were no cells in the series exposed to ^3H -thymidine for up to 6 hours which were doubly labeled; no cell that had grains above its nucleus also bound the antimyosin. There were, however, a small number of cells in the series exposed to ^3H -thymidine for 10 hours, and more cells in the 15-hour series, which were doubly labeled. In these cells the S period is 5 hours, G₂ 1.5 hours, and M 1 hour. Therefore it follows that myosin is not synthesized in detectable quantities in S, G₂, M, or even in the first few hours of G₁.

From these data I would argue that the replicating, presumptive myoblast is a stem cell. As a stem cell fabricating the various kinds of molecules required for cell division, it is precluded from activating the machinery required to turn out myosin. As a stem cell the presumptive myoblast is not a naïve, uninstructed, "undifferentiated" cell. On the contrary, in a fashion as yet unknown the muscle precursor cell must be different from all other cells. At some point in its mitotic history the presumptive myoblast undergoes a "critical" division and one or both daughter cells begin to translate for myosin for the first time. Before this "critical division" and the subsequent 5-hour period in G₁, myogenic cells do not make small amounts of myosin, hemoglobin, or other comparable molecules; during this period the stem cells are concentrating on maintaining their myogenic precursor nature. Nothing is currently known of the unique molecules which the myogenic stem cells must synthesize to maintain this state. Similarly, how multiplying presumptive myoblasts propagate their myogenic information, or how a "critical" mitotic division results in a daughter cell which withdraws from the mitotic cycle and becomes a myosin-synthesizing and/or fusing cell are problems we may still be discussing at the next Review Conference.

Dr. Herrmann: In principle, I quite agree with what has been said. You realize from my talk that our interpretations are based on what we (after Dr. Holtzer's work) regard as fact—namely, that myosin production and DNA synthesis in the developing muscle cells are mutually exclusive. I hope it is so because, otherwise, all our results must be revised.

Nevertheless, there is one question I would like to raise. Can we be sure that those few cells having G₁'s much longer than 7 hours (which we have in the developing embryo) will not make small amounts of myosin? We have to assume there are periods when these cells are not getting ready, do not produce thymidine kinase, DNA polymerase, and all the rest of the enzymatic inventory necessary for DNA synthesis.

I have no evidence one way or the other, but we have to realize we have cells, which eventually will still participate in the proliferation, that have G₁ periods much longer than 7 hours.

Dr. Holtzer: Our evidence only permits us to say that a minimum of approximately 5 hours in G₁ elapses before myosin is detected in cells that will no longer enter S. Most cells, however, spend 10 or more hours in G₁ before myosin can be detected. I would like to know if a cell stays in G₁ for, say, 2 days; does it remain properly programmed for myosin synthesis or must, or can, it go through another cell division?

How can the G_1 leading to myosin synthesis be distinguished from the G_1 leading to DNA synthesis? These are exciting questions about which I have little to offer.

Dr. Herrmann: So, as long as the cell will eventually get ready to proliferate, to divide, in your interpretation, it will not form myosin no matter how long the G_1 period is. Is that right?

Dr. Holtzer: Quite right.

Dr. Harary: I would just like to say that your results would preclude that a cell which has made myosin can be myogenic because you never find the two together. It has been shown by Kasten and by Marks that these cells, being cells, can divide. Cells that have myosin can divide. Therefore there is some evidence that cells can synthesize DNA while they contain myosin in the cytoplasm. This doesn't mean they are doing it at the same time. They could alternate in this process. But I wonder why in your system you haven't found cells that go through this cycle and whether it is just a matter of the time period you have chosen.

Dr. Konigsberg: I wonder whether there isn't some misunderstanding about this 7 hours. This 7 hours represents the minimum amount of time that it takes before you get a labeled nucleus into a myotube. I mean, essentially, your cells don't have the opportunity to recycle.

Dr. Holtzer: There is a minimum amount of time a cell must spend in G_1 after the "critical mitosis" both for the synthesis of myosin to begin and for the acquisition of the ability to fuse.

With respect to the comments of Dr. Harary, several years ago we (Holtzer, Abbott, and Cavanaugh, 1959) described growing cultures of cardiac cells, some of which contained myosin. What we did not observe in those cultures, which were about doubling in number in 24 hours, was a single cell with both a normal mitotic figure *and* a normal complement of myofibrils. If Dr. Harary knows of results contrary to these findings, I would appreciate learning about them. For a number of reasons, it is theoretically conceivable that a cell may be induced to cease functioning as a muscle cell, cease synthesizing myosin, and to reenter the mitotic cycle. For several years we have been seeking evidence for this type of transformation but as yet have not been successful. By the way of contrast, a functioning chondrocyte is readily transformed or induced to become a dividing cell.

As for the movie by Marks and the evidence of Kasten, I would say they have not demonstrated that a beating heart cell divides or that cells with myofibrils contain normal mitotic figures. Anyone who has cultured beating heart cells knows that very often fibroblasts adhere to contracting cells. Under these conditions the fibroblasts appear to beat. These fibroblasts may divide. In my opinion, the sequence in the movie by Dr. Marks shows a dividing fibroblast adhering to beating heart cells. If in fact this were not so, it should be very easy by simple conventional staining to demonstrate many cells with mitotic figures *and* myofibrils. The very fact there are many reports of *amitosis* in cells with myofibrils, but very few, if any, unequivocal photographs of mitosis in cells with myofibrils, makes it unlikely that the normal cardiac cell with striations can divide.

Dr. Herrmann: I would like to introduce a different angle to both Dr. Harary's and Dr. Konigsberg's observations. I think, when you put differentiated muscle fibers into culture, they begin to leak. Quite possibly, in response to the loss of cell constituents, they turn on their synthetic processes. Can anything prevent this leakage, or is perhaps innervation, which is necessary for continued muscle differentiation, a factor in tightening the muscle cell membrane?

Dr. Harary: On the question of leakiness of the cells, we are all aware that a cell treated with trypsin and placed in culture is leaky. But they do recover. They do repair.

We have given some attention to repair of the membranes in these cells to determine what relationship this has to their function. With linoleic acid added to a medium containing no lipids, one can maintain mitochondrial function, mitochondrial membranes (in an intact state), and also cell membranes, as measured by amino acid activity, amino acid uptake (in an intact state), and yet show dedifferentiation in these systems. Moreover, one can show that lipids have an effect on reversing with dedifferentiation. This may be part of the answer.

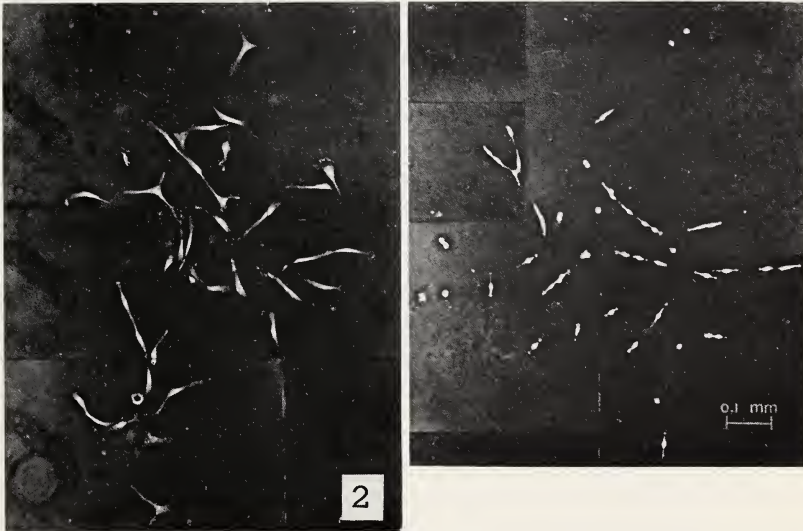
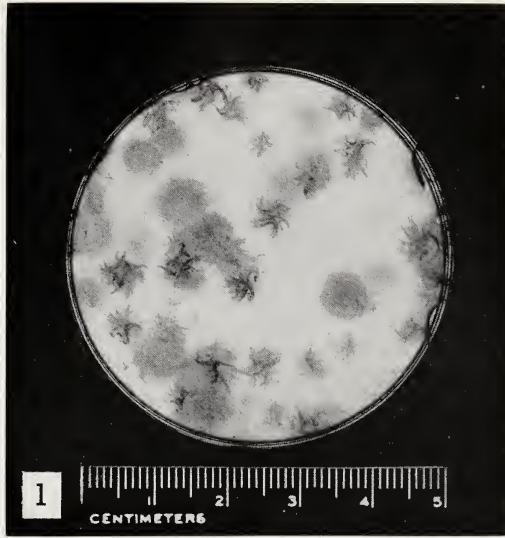


FIGURE 1.—Culture initiated with 200 cells from the leg musculature. Fixed and stained after 13 days of cultivation. Irregular-shaped colonies with fibrous appearance are muscle colonies containing cross-striated multinucleated myotubes.

FIGURE 2.—Colonies photographed with phase optics on the 4th day in culture. Colony *on right* developed into a muscle colony; the one *on left* is a colony of "fibroblastic" cells.

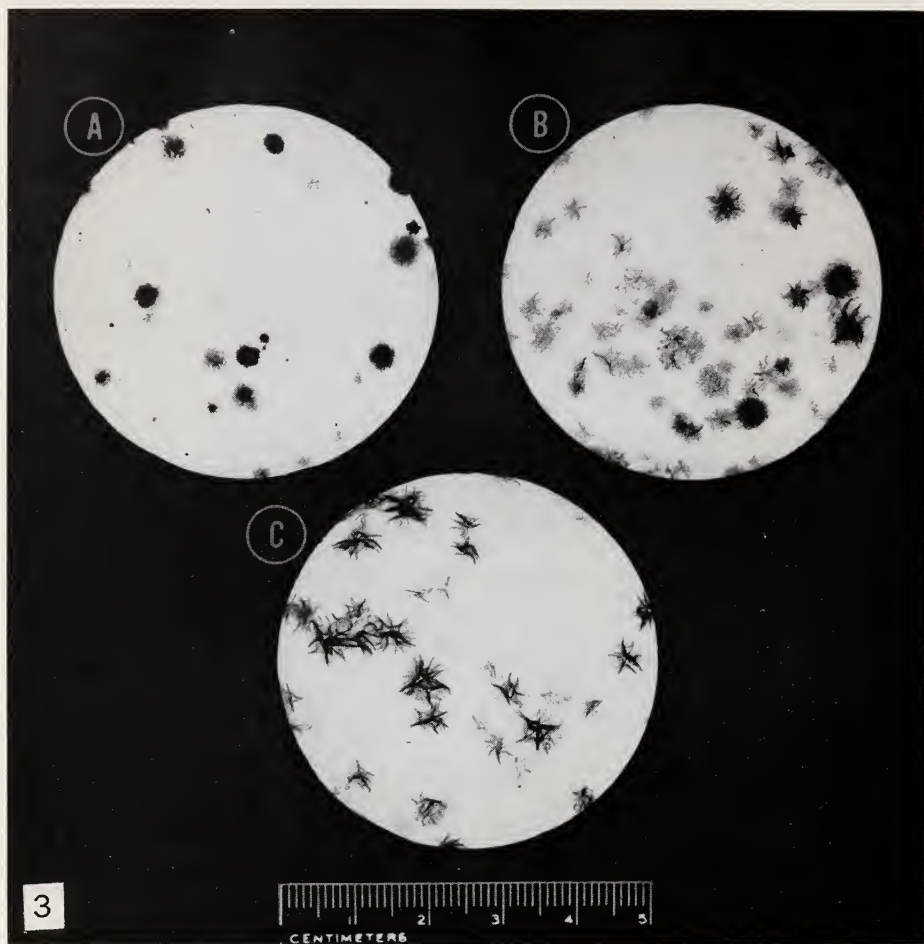


FIGURE 3.—Replacement of conditioned medium with collagen substratum. Colonies cultivated on: unconditioned medium (A), conditioned medium (B), and on a film of precipitated collagen in the presence of unconditioned medium (C).

The Single-Active-X: Functional Differentiation at the Chromosome Level^{1, 2, 3}

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SUMMARY

Human cells, especially fibroblasts, exhibit all major aspects of the single-active-X control scheme during prolonged cultivation *in vitro*. Only one glucose-6-phosphate dehydrogenase allele of the two present in diplo-X cells is expressed in any clone. The X's with unexpressed genes form sex chromatin, are late replicating, and produce replicas having the same properties. The cells can be useful in detecting reversibility of the control scheme, especially if they have morphologically characteristic

X chromosomes or genes with phenotypes detectable at the single cell level. The control scheme may now be amenable to study in cleaving embryos. Mouse embryos having 7–17 cells when removed from the oviducts do not have clearly identifiable sex chromatin bodies. The embryos continue to develop when cultured *in vitro* and about one half of the embryos with 50 or more cells have distinct sex chromatin bodies.—*Nat Cancer Inst Monogr* 26: 327–351, 1967.

MAJOR APPLICATIONS of the *in vitro* culture technique will increasingly concern studies of differentiation or, more generally, the regulation of genic expression in complex organisms. I will discuss an example of regulation that has particular relevance to the theme of this Decennial Review Conference—the correspondence, or lack of it, between cells *in vitro* and their *in vivo* progenitors. This is the “single-active-X” control scheme (1, 2), which insures that genes on only one X chromosome are phenotypically expressed in mammalian somatic cells that have more than one X. The scheme is important in mammals because it achieves a parity in the quantitative expression of certain X-linked genes in males having

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

² Supported by Public Health Service research grants GM-08217 and GM-06983 from the National Institute of General Medical Sciences.

³ Paper No. 1118 from the Laboratory of Genetics of the University of Wisconsin.

one X and females having two of them. An understanding of how such "dosage compensation" (3, 4) is achieved would, alone, make study of this control scheme very worthwhile. You will see that the control involves several major aspects of chromosome behavior: physical state, the timing of replication, and genic expression. We may also gain some insight into the behavior of chromosomes in general.

The available evidence indicates that all major aspects of the control scheme that are manifested *in vivo* are also manifested *in vitro*. Therefore, a cursory description of the phenomenon only as it appears in cultivated cells will serve our purpose well enough. The *in vivo* corollaries and most of the details, as well as necessary qualifications and exceptions to statements written below, can be found in references (5-7).

OUTLINE OF THE SINGLE-ACTIVE-X PHENOMENON

Early in the development of diplo-X embryos the X's become differentiated into two functional types, "active" (X_a) and "inactive" (X_i), that have several contrasting attributes. It is convenient to relate them to the different stages of the cell cycle.

Attribute	"Active"	"Inactive"
Forms sex chromatin body during interphase (fig. 1A, 1B)	No	Yes
Replicates mainly near end of S (fig. 1C)	No	Yes
Allocyclic condensation during mitosis (fig. 2A, 2B)	No	Yes
Genic expression	Yes	No

During interphase, portions (it is not known exactly which portions) of each X_i may form an individual, densely staining chromatin condensate, the Barr, or sex chromatin body (fig. 1A). The incidence of cells with distinct Barr bodies varies with the stage of interphase and with the growth rate of human fibroblast populations (8-10). Possibly, every diplo-X cell has the capacity to form a sex chromatin body since the Barr bodies are found in more than 90% of the nuclei of populations grown to maximum density. There is only one active X per cell. All others, as many as four in some humans, remain inactive. The cytological manifestation of this all-X's-but-one rule is that the maximum number of Barr bodies per diploid nucleus is one less than the total number of X chromosomes (fig. 1B).

Autoradiographic, "late-labeling" experiments show that X_i replicates *mainly* during the last hour or 2 of the DNA synthetic portion of the cell

cycle. Radioactive DNA precursor given at this time results in major, but usually not exclusive, labeling of X_1 as studied at the subsequent metaphase. As an alternative, it may prove useful for some purposes to give a brief pulse exposure to radioactive precursor earlier in S, for this results in significant labeling of all chromosomes but X_1 (fig. 1C).

The allocyclic condensation of X_1 is also manifested as precocious condensation in early prophase (fig. 2A) but this relation is reversed at metaphase. For then X_1 has a distinctly "fuzzy," decondensed appearance (fig. 2B) when prepared for examination in certain ways (11, 12). It is not known if the fuzziness reflects the native state of the chromosome or if it is the reaction of a chemically distinctive X_1 to the preparative procedures.

The active X is ordinarily chosen at random from among the X's present in a cell. The paternal X ($^P X$) becomes active in some cells of the embryo while the maternal X ($^M X$) does so in others. These initial determinations are "fixed": Each cell in which such a determination is made generates a clone in all cells of which the same type of X, either of maternal or paternal origin, is active. When X_a and X_1 have the same type of allele at a locus, the net result is quantitative expression corresponding to one X. When the two X's have different alleles at a locus, individual clones have the phenotype corresponding to one allele or the other, but not to both. Two laboratories (13, 14) have demonstrated that clones grown from single human fibroblasts heterozygous for alleles affecting glucose-6-phosphate dehydrogenase (G6PD) exhibit such single-allele expression.

The cytological counterpart of this result in cultured fibroblasts is the evidence that X's which form Barr bodies during one replication cycle do so in succeeding replication cycles (15).

The single-active-X phenomenon is carefully documented in many ways, both *in vivo* and *in vitro*, but no one knows how it works. Not enough is known to form an over-all theory that makes predictions amenable to critical experimental test (16, 17). I will merely present an outline of commentary, ideas, and experiments. Many will conclude, with me, that the proposal made below is an *ad hoc* contrivance made up of fuzzy notions about the fuzzy X. My own attitude was aptly described by the anthropologist, Gladwin. He proposed that it was the sailors of Alexander the Great, and their amalgam with the Melanesian-Carib and Polynesian-Arawak, who sailed across the Pacific and sparked the advance of Indian civilization in mid-America (18).

We are going to offer an explanation that will be a radical departure from those in current circulation, and I shall be the first to admit that this tale will need a great deal of patching and strengthening before it will carry much weight. This may seem a strange way to launch a new theory, but I am more concerned in opening up new channels of inquiry than in trying to provide pat

answers to all the questions that are plaguing us . . . and it is for this reason that I have said that this tale will need support and will undoubtedly need to be changed. This, however, is the way that every new theory should be treated, and no harm will be done if, when a new idea is launched it is regarded with due reserve, but also without prejudice.

PROPOSAL FOR A SINGLE-ACTIVE-X CONTROL SCHEME

General comment.—It may be useful to set the single-active-X in a roughly defined spectrum of repression schemes. At one extreme might be placed the generally condensed chromatin of thymocytes. This exemplifies a rather coarse kind of control, under which both general replication and genic expression are repressed (19). The opposite end of the spectrum is occupied by the operon type of control found in microbes. Single, or small groups of structural genes are controlled by specific regulatory genes that turn them "ON" and "OFF." This type of fine control permits replication to proceed whether extensive transcription occurs or not.

The single-active-X falls somewhere between these extremes. X_1 replicates during each cell cycle, although its phenotypic expression is repressed. It is condensed during portions of interphase but represents only about 2–3% of the chromatin in a diplo-X cell, while closer to 80% is condensed in thymocytes. Even so, the human X has roughly 10 *Escherichia coli*-equivalent amounts of DNA. Assuming a unineme structure, this corresponds to several thousand genes. If a reasonably low degree of polynemy is assumed, quite a large number of genes with many unrelated functions is still involved. There are also reasons for thinking that chromosomes consist of a number of different DNA molecules or portions of such molecules that are individually regulated for replication purposes and arranged in some sort of tandem array (20). Numerous units of regulation for transcription are also suggested by this, but the human X_1 is *generally* fuzzy at metaphase and its over-all replicative activity is almost nil during most of S. The chromosome seems to be regulated as a unit, but one that is too large to fit a simple operon-type scheme. The notion of unitary control is strengthened by the findings that autosomal genes which are translocated to the X can be repressed along with the remainder of X_1 (2, 21).

There is at least one microbial example in which numerous genes carrying out diverse functions and occupying a single "large" genetic structure are repressible as a unit. The genome of the temperate phage, λ , can exist in two different states:

(a) *vegetative*, in which the viral genome replicates repeatedly after its invasion of the host bacterium. All functions necessary for the production of a new crop of mature infectious virus particles are executed.

This is done by about 30 genes directing the production of proteins as diverse as lysozymes, synthetic enzymes, exonuclease, tail fibers, and head capsomeres. Yet all of these functions, including unrestricted replication, are repressible under the influence of one, or a few, genes in the *c* region of the genome (22), especially when the genome is in its alternative state.

(b) *prophage*, in which the λ genome is physically and functionally integrated into the genome of the host bacterium. In this state it takes its turn in the ordered replication of the bacterial chromosome, doubling once during each bacterial replication cycle. Expressions of its genes for vegetative functions are repressed. More than this, the presence of an integrated prophage in a bacterium represses both the unrestricted replication and vegetative functions of additional λ genomes introduced into the same bacterium. This repressive influence is transmitted through the cytoplasm.

The *c* region may be effective through interaction of its product(s) with the *c* region of other λ genomes, repressing a step necessary for initiating extensive replication and transcription (22). There is no equally explicit evidence for such chromosome-specific, sequential relations in mammalian cells right now. Specific assumptions about such steps are not essential to my purpose. The formal analogy with the single-active-X is obvious and may finally prove to be relevant down to details. For the moment, it merely describes a set of relations that may apply to X chromosomes but explains nothing in physical or chemical terms.

X-coiler.—We propose the existence of a gene or genes concerned with the production of a substance or substances that will be referred to as “X-coiler.” X-coiler effects the elementary process of repression, which is to induce coiling along X_1 (but not X_a or autosomes) in a manner that prevents replication and transcription. This coiling is not to be confused with the obvious condensation that is manifested as the Barr body. Coiling may still be completely repressive, although not detectable in the light microscope. At least three laboratories have reported that distinct Barr bodies are not detected in many rapidly proliferating, diplo-X fibroblasts (8–10), but it is with just such cells that the clonal nature of single-allele expression has been demonstrated (13, 14). Electron microscopic examination of thinly sectioned Barr bodies in human fibroblasts reveals a rather diffuse network of coiled chromatin fibrils that would, individually, be invisible in the light microscope, rather than the very dense packing characteristic of metaphase chromosomes or thymocyte nuclei (23). This suggests that obvious condensation is often an accompaniment of, but not a condition for, X-chromosome repression. It may be a direct, although not invariable, consequence of prior coiling or may involve additional processes. In the latter case, one may encounter X_1 ’s that coil but do not condense as natural variations between different species or as abnormal variants within a species.

It is simple to place the genes directing the production of X-coiler on the X chromosomes themselves, although workable schemes with autosomal loci are easily contrived. We will assume a locus on the X and leave the chemical nature of X-coiler as a completely open question.

The X-coiler cycle in somatic cells.—The X-coiler genes on X_1 may or may not be repressed along with other genes, but the gene on X_a directs the production of X-coiler at the beginning of interphase. As a consequence, replication and transcription of genes on X_1 are repressed. Late in S, "signals" are generated dictating the modification, removal, or destruction of X-coiler. These signals could be enzymes or enzyme products. They interact with many sites on X_1 , evoking a generalized derepression of replication, and transcription for a relatively brief interval of time. The X-coiler gene on X_1 could be expressed at this time, leading to the coiling and condensation of X_1 that regularly follows its replication, *i.e.*, both chromatids must be repressed during G_2 . The restricted transcription on X_1 , which is postulated here, is of a magnitude that might be detected by some phenotypic criteria and not by others. The sensitive concentration dependence of some regulatory schemes indicates that even the limited transcription postulated here—say, 5–10% of X_a —could contribute to the difference between XO and XX phenotypes. It might explain the fact that all the red cells of individuals heterozygous for the X-linked gene, g_a , which determines a surface antigen, have "+" reactivity (24). The surface antigens are relatively far more persistent than many enzymes during the 130-day average lifespan of the enucleate red cell. The small increment of G6PD, above the level due to X_a , that could result from the last transcriptions on X_1 in a red cell precursor could hardly be detected by ordinary quantitative determinations (25). In contrast, the small amounts of $g_a(+)$ antigen resulting from the last transcriptions in a cell that was $X_{1a}^{(+)} / X_{1a}^{(-)}$ could confer a persistently "+" phenotype.

Since a generalized chemical difference between X_1 and X_a has been proposed, the fuzzy metaphase X_1 can be interpreted as a differential response of the chromosome to the heat or acid treatment used to evoke the fuzziness (11, 12). Alternatively, it is possible that X-coiler is removed or altered during mitosis. This could also explain the fuzziness and would require synthesis of new X-coiler early in interphase, as proposed above.

Initiation of the X-coiler cycle in somatic cells.— ^MX and ^PX can be either X_1 or X_a in different clones derived from a single zygote. The hypothesis of randomness postulates that both X's are equally reactive in the initial differentiative events. The question is whether both X's are initially "ON," *i.e.*, active, or initially "OFF," *i.e.*, inactive. There are at least two reasons for thinking that, mainly, both are initially "OFF."

In considering humans with 5 X's per cell, it is easier to think of X chromosome differentiation as turning 1 of 5 initially inactive X's "ON" than of turning precisely 4 of 5 initially active X's "OFF."

Prior to late cleavage, there is relatively little synthesis of messenger RNA in sea urchin embryos (26), or of 4S (transfer?) and ribosomal RNA in embryos of *Xenopus laevis* (27). Many genes seem to be "OFF" as far as transcription or translation are concerned, although replication proceeds rapidly. Barr bodies make their first appearance in mammalian embryos in the late blastula stage, at about the same time as extensive transcription and translation might be expected to begin. In keeping with this is the finding that the G6PD activity of mouse embryos is the same during the first 3 days of development as it is in the mature, unfertilized ovum (unpublished results). The initial differentiative events could occur in the context of the general activation of the genome at about the time of gastrulation. By chance, one of the X's, either maternal or paternal, could be activated by generalized chemical alteration. This activation would be of the sort that activates the autosomes. It would, at once, derepress the X-coiler gene of X_a and make the chromosome immune to the repressive action of X-coiler. X's not yet activated at this time would then interact with X-coiler. Having combined with X-coiler, these chromosomes would no longer be susceptible to activation of the autosomal type and would become the X_1 's. This initiates the X-coiler cycle outlined above and requires at least two things:

(a) that chromosomes undergo generalized chemical changes at various times. Examples of this kind of generalized alteration are the transitions from histone to protamine or to arginine-rich histone that occur during spermatogenesis in many organisms, including mammals. The original type of histone-rich chromatin must be reconstituted during cleavage in these cases.

(b) that both of the X chromosomes be specifically susceptible to interaction with X-coiler during cleavage and that the autosomes ordinarily lack such susceptibility. The X-specific susceptibility could arise during meiosis if the X chromosomes occupy a microenvironment in which they acquire a generalized chemical difference from the other chromosomes. An example of such a microenvironment would be the sex vesicle associated with the XY pair during spermatogenesis. The proposal requires that similar chemical differences between the X and autosomes arise during oogenesis, but an entity analogous to the sex vesicle has not yet been described for this process.

There is another reason for giving serious consideration to meiotic processes involved in X-chromosome differentiation. It is conceivable that the generalized susceptibility of the X to X-specific repression could have evolved as an attribute of genes on the X. It is difficult to see how an autosomal fragment, not having evolved in this respect, can be repressed if translocated to X_1 . It is easier to imagine this if the autosomal fragment acquires its susceptibility to repression along with the rest of the X during meiotic preconditioning.

SOME EXPERIMENTAL APPROACHES

The proposal outlined above is necessarily vague and still permits contradictory predictions. Appropriate predictions, as well as experimental treatments suitable for altering the behavior of X_1 , will be obvious to many. I will merely sketch out some experiments, emphasizing the *in vitro* technique, which are feasible now and which should prove helpful in refining notions about the X. These will be outlined in the context of two general approaches:

(a) interfering with the control scheme after it has become operative in somatic cells;

(b) interfering with the initial differentiative events in embryos in ways that give clues to their nature.

Detecting activation of the inactive X.—It has already been proposed that genes on X_1 are expressed to a limited extent at the end of S and one might start by attempting to detect this baseline expression without attempting disruption of the control. Relevant suggestions have been made by us previously [p. 45 (14)] but they can be refined now. The G6PD genes are under the control scheme in cultivated human fibroblasts. It should be possible to produce synchronous populations of such cells that might show a small increment in enzyme activity near the end of S. The size of this increment should be at least roughly proportional to the number of X_1 's present and should be zero in haplo-X populations. Attempts to synchronize diploid human fibroblasts on a large scale have not been very successful, but populations in the range of 10^4 – 10^5 cells can readily be obtained by simply tapping loose the dividing cells in unsynchronized cultures. Small samples of such populations can be placed on slivers of coverslips, where they quickly reattach and resume progress through the cell cycle. One can then conveniently make quantitative determinations of their G6PD activity by merely air-drying the cells *in situ*, placing the coverslip and the G6PD reaction mixture in a cuvette, and following the appearance of TPNH using a fluorometer. This method (to be published) yields as much enzyme activity as can be detected by any standard procedure for cell disruption and is applicable to less than 10^3 cells without special modifications of ordinary fluorometer equipment.

The proposal outlined suggests that should interaction of X_1 with X-coiler be replaced, partially or totally, by interaction with autosomal types of activator the behavior of X_1 should be permanently altered in several respects. One of these would be expression of genes that previously were unexpressed. There is no information on the viability of individuals or cells that are diplo-active-X, but a number of human aneuploid states that are extremely deleterious *in vivo* have little deleterious effect, if any, on cells *in vitro*. There is a good chance that diplo-active-X cells would be fully viable and the problem lies in creating and then detecting them. Some reports that cells lines of diplo-X origin no longer form Barr bodies [(28),

for example] can't be interpreted as conclusive evidence for the presence of two active X's. In no instance has the presence of two X's or the simultaneous expression of two alleles of an X-linked locus been established.

We made the pessimistic assumption that reactivation of a previously unexpressed G6PD allele on X_1 would, in the beginning, occur with low probability and require an extremely sensitive detection system. Direct selection of infrequent variants, as for drug-resistant mutants, has not been successfully applied to G6PD mutants. Visual selection permits a solution to the technical problem in the following way. Fibroblasts with normal levels of G6PD reduce tetrazolium salts under appropriate conditions and acquire conspicuous, insoluble deposits of formazan, but mutant cells that are sufficiently deficient in G6PD fail to do so (29). The study of artificial mixtures of normal and mutant cells indicates that it should not be difficult to detect a 10^{-6} incidence of G6PD (+) cells in a (—) population (fig. 3). Application of this technique to phenotypically (—) clones derived from a +/— heterozygote, *i.e.*, clones that are X_1^+/X_2^- could permit detection of cells in which the (+) allele has been reactivated. So far, all the clones derived from one such heterozygote have proved to be (+) but this should not remain the case (13).

Activation of X_1 could be manifested as altered time of replication. Since the background labeling of X_1 is so low following a brief radioactive pulse at mid-S (fig. 1C), this experimental format might permit maximally sensitive detection of earlier-than-normal replication. The frequently nondescript morphology of the human X could impair the interpretation of such experiments and it might be desirable to use morphologically distinctive X_1 's. The X chromosome of cultured Chinese hamster cells is easily identified and the labeling time of the normally late-replicating arms could be related to that of the arm which normally replicates earlier (30). As an alternative, morphologically distinctive human X_1 's are available. Figure 4A depicts the metaphase of a cell that is heterozygous for a normal X and a structurally aberrant X that is X_1 in all cells of a fibroblast culture. It forms a distinctive Barr body (fig. 4B). If this strain yields derivatives that fail to form sex chromatin, it should not be difficult to verify persistence of the original chromosome.

Studying the initial events in embryonic cells.—It is becoming increasingly practical to cultivate cleaving, mammalian embryos. A suitable indicator of X-chromosome activity is needed if this *in vitro* tool is to be applied to the single-active-X problem. Experiments involving treatment of embryos during *in vitro* sojourn followed by their continued development within "incubator" females are practical now (31). Both cytogenetic and phenotypic criteria, *e.g.*, coat color, could be used for scoring X-chromosome behavior in the resultant embryos or full-term mice. I will merely report here what may be a first step toward a completely *in vitro* system. Mr. Harold Rehbein, a medical student, and I have been able to detect the

early appearance and subsequent increase of cells with Barr bodies in cultivated, cleaving mouse embryos.

Ovulation was induced in randombred, white Swiss mice, using Edwards' technique (32). The females were mated on the night when ovulation occurred and that midnight is taken as zero time here. We obtained embryos at approximately 60–65 hours by slitting the oviducts in culture medium (33) or by flushing several ml of medium through them. Some embryos ("ductal") were fixed immediately in ethanol-acetic acid (3:1, v/v) to determine the initial cell numbers. Others ("cultured") were transferred through fresh culture medium with Pasteur pipettes and then placed singly in 0.05 ml drops of medium on coverslips under 3.5 ml of Bayol 55 oil in 35 mm Falcon plastic dishes. No maternal cell contaminants were detected microscopically. The cultures were incubated at 37 C in a humidified atmosphere containing 5% CO₂ in air and were fixed in "3:1" after either 4 or 7 days. The number of nuclei and the presence of sex chromatin were determined after staining with the Feulgen reaction. We made the same two determinations with the ductal embryos by transferring them individually from fixative to drops of lactic-acetic orcein (34) on a slide and placing a coverslip over them to minimize squashing and shearing.

The number of cells in 27 ductal embryos ranged from 7–17 (average 13.7), excluding polar bodies. Hatching from the zonae pellucidae began after 1 day in culture, during the 4th day after ovulation. The embryos then attached to the glass and flattened out to varying degrees. Mitoses were evident in some cultures after 7 days in culture.

Table 1 summarizes nuclear counts and observations of sex chromatin in cultured embryos. Standards for scoring sex chromatin were cells cultured from 22 11-day embryos, fixed and stained as were the younger embryos. In each case, karyotype analysis confirmed prior sex chromatin diagnosis (fig. 5A, 5B).

According to these standards, the cultured embryo shown in figure 5C is male and in figure 5D is female. Fourteen of the 94 nuclei (15%) contained a heteropyknotic mass judged to be sex chromatin and 6 of these are shown in figure 5E through 5J. Sex chromatin was found both in the peripheral nuclei (fig. 5E, 5F), which tend to be flattened and of relatively large cross-section, and in the smaller appearing, more rounded, and centrally located nuclei (fig. 5G through 5J).

In comparison, the cultured embryo shown in figure 5C is presumed to be male. None of the 96 nuclei present contained a distinct sex chromatin body. The two embryos illustrated were chosen because they had a similar number of nuclei, most of which had unclumped chromatin. A distinct incidence of apparently polyploid nuclei is evident in the embryo in figure 5D but the great majority of all nuclei with sex chromatin were apparently diploid.

TABLE 1.—The number of nuclei and the presence of sex chromatin in cultured mouse embryos

Number		Sex chromatin		
Nuclei per embryo	Embryos*	+	—	?†
21-30.....	3	1	1	1
31-40.....	1	1	0	0
41-50.....	6	1	2	3
51-60.....	3	2	0	1
61-70.....	3	0	1	2
71-80.....	5	1	3	1
81-90.....	7	4	3	0
91-100.....	5	2	3	0
101-110.....	5	1	2	2
111-201.....	8	3	1	4
Total.....	46	16	16	14

* The embryos were derived from 9 females, from 3 of which the uncultured "ductal" embryos were also taken (see text).

† Nuclear counts could be made but technical quality did not permit reliable scoring of sex chromatin.

Each of two 59-cell cultured embryos had five nuclei (8.5%) with sex chromatin, but the scoring of smaller embryos as female was based on only two or three nuclei with presumed sex chromatin in each case. Independent methods of sexing these smaller embryos will be necessary to establish scoring accuracy. The maximum incidence of sex chromatin detected so far is 20% in an embryo culture containing 183 nuclei. It is unlikely that another embryo containing 167 nuclei would have the same chromosomal makeup, but, by chance, no definite suggestion of a Barr body.

Another reason for believing that the classes of cultured embryos defined by sex-chromatin analysis correspond to a sex difference is the occurrence of equal numbers in the positive and negative groups. We can't assume a primary sex ratio of exactly 1:1 in the mouse but neither is it likely to be very different from that value.

We have been unable to separate the ductal embryos of 7-17 cells into two classes defined by sex chromatin analysis. All nuclei of some ductal embryos lacked a clearly suggested Barr body. Others had several nuclei with a general, coarse clumping of the chromatin, which made it difficult to identify sex chromatin. Other techniques will be needed to determine the state of the X chromosomes in such early cells. Our observations can be explained by either of two alternatives that should soon be experimentally resolved:

1) If the Barr bodies observed in cultured embryos result only from events that occur before *in vitro* culture, then X-chromosome differentiation begins by the 17-cell stage in the mouse.

2) X-chromosome differentiation can occur after the 17-cell stage *in vitro*.

We believe that sex chromatin is present by the 50-60 cell stage of female mouse embryos. These are far fewer cells than the 500 present at its first detection in the rabbit (35), or the cat, man, and monkey (36, 37). However, all previous reports agree regarding the first detection of sex chromatin at the blastocyst stage, corresponding *roughly* to 50 cells in the mouse.

Sex chromatin can be a relatively insensitive indicator of functional differentiation. It is conceivable then that functional X-chromosome differentiation precedes the first appearance of Barr bodies and is operative in embryonic cells lacking them. None of the results to date decisively exclude the possibility that the first cleavage division results in one cell with the paternal X active and a sister with an active maternal X. The various proportions of these two types of cells could subsequently result from combinations of selective and random effects. Alternatively, Barr-body-less embryonic nuclei may actually indicate an absence of X-chromosome differentiation. The current, probably temporary, lack of a useful X-linked marker that can be studied at the cellular level in the mouse need not prove an insuperable barrier to tackling such problems with this animal. In the meantime, karyotypic and sex-chromatin analysis of variously treated embryonic cells may allow us to make some headway. I feel that this particular aspect of our work is quite close to the spirit of this Conference. While it is concerned with work that has been in progress for several years, it is clearly the beginning rather than the end of something.

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PLATES

PLATE 48

FIGURE 1A.—Nucleus of a cultivated human fibroblast stained by the Feulgen reaction. The nucleus is diploid and diplo-X and has a single Barr (sex chromatin) body.

FIGURE 1B.—Like A, but the cell was cultured from a human with four X chromosomes per cell. This nucleus has three Barr bodies.

FIGURE 1C.—The metaphase of a cultured, diplo-X, human fibroblast that was given a 2½-minute pulse exposure to tritiated thymidine [$5/\mu\text{c}/\text{ml}$, 10.6 c/m mole, in medium F4 (33)] about 10 hours before it entered mitosis. Presumably the cell was in about its 4th hour of S at the time of the pulse. *Arrow* indicates the X chromosome that was not replicating appreciably at this stage of the cell cycle.

Autoradiography and photography performed by Miss Shirley Brody of this laboratory.

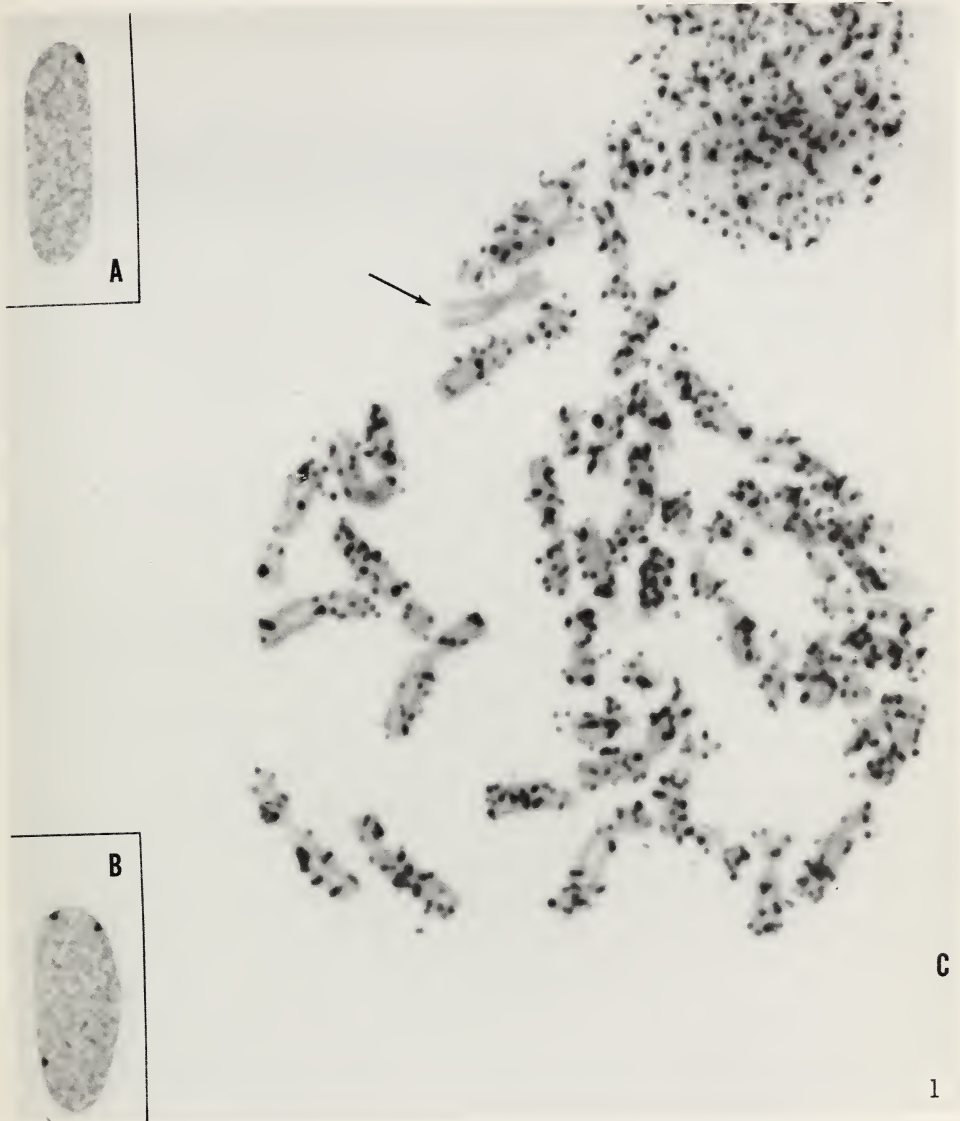


PLATE 49

FIGURE 2A.—Early prophase of a cultured, diplo-X, human fibroblast. The cell was grown on a coverslip, fixed *in situ* with ethanol-acetic acid (3 : 1), squashed, and stained with the Feulgen reaction. *Arrow* indicates the X that is relatively condensed compared to the other chromosomes.

FIGURE 2B.—Metaphase of a diplo-X fibroblast prepared according to our procedure as described in (12). *Arrow* indicates the "fuzzy" X that appears to be relatively decondensed, compared to the other chromosomes, at this stage of mitosis. Lactic-acetic orcein stain (34).

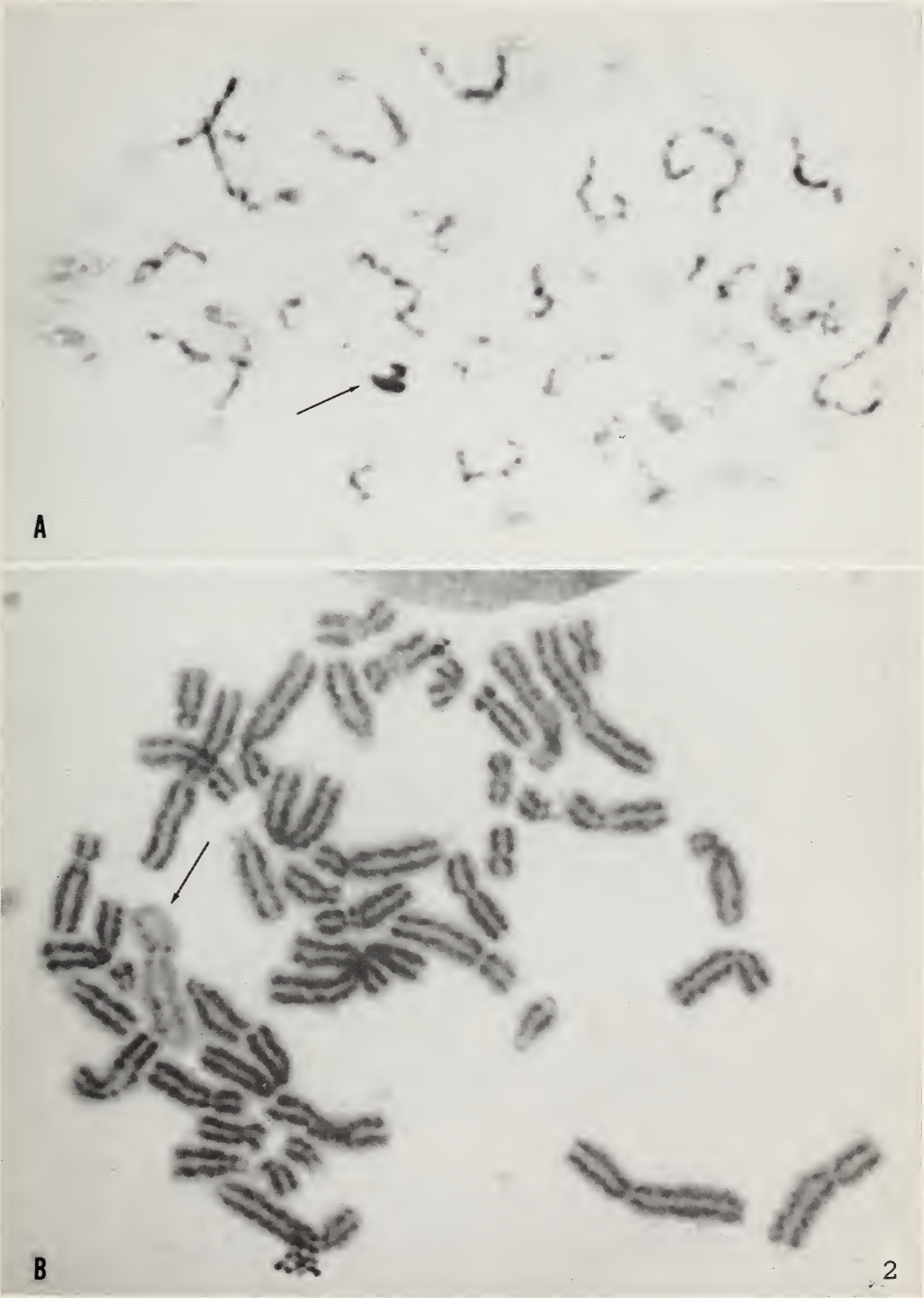




FIGURE 3.—A single, cultured human fibroblast with normal glucose-6-phosphate dehydrogenase activity (*heavy arrow*) amid many fibroblasts cultured from a human with a mutation causing a deficiency of the enzyme. An artificial mixture of mutant (majority) and normal (minority) cells was cultivated on a coverslip and then treated according to Wajntal and DeMars (29). The normal cell reduced tetrazolium and had conspicuous deposits of formazan. The mutant cells failed to reduce the tetrazolium and were made visible by counterstaining their nuclei with methyl green (*small arrow*).

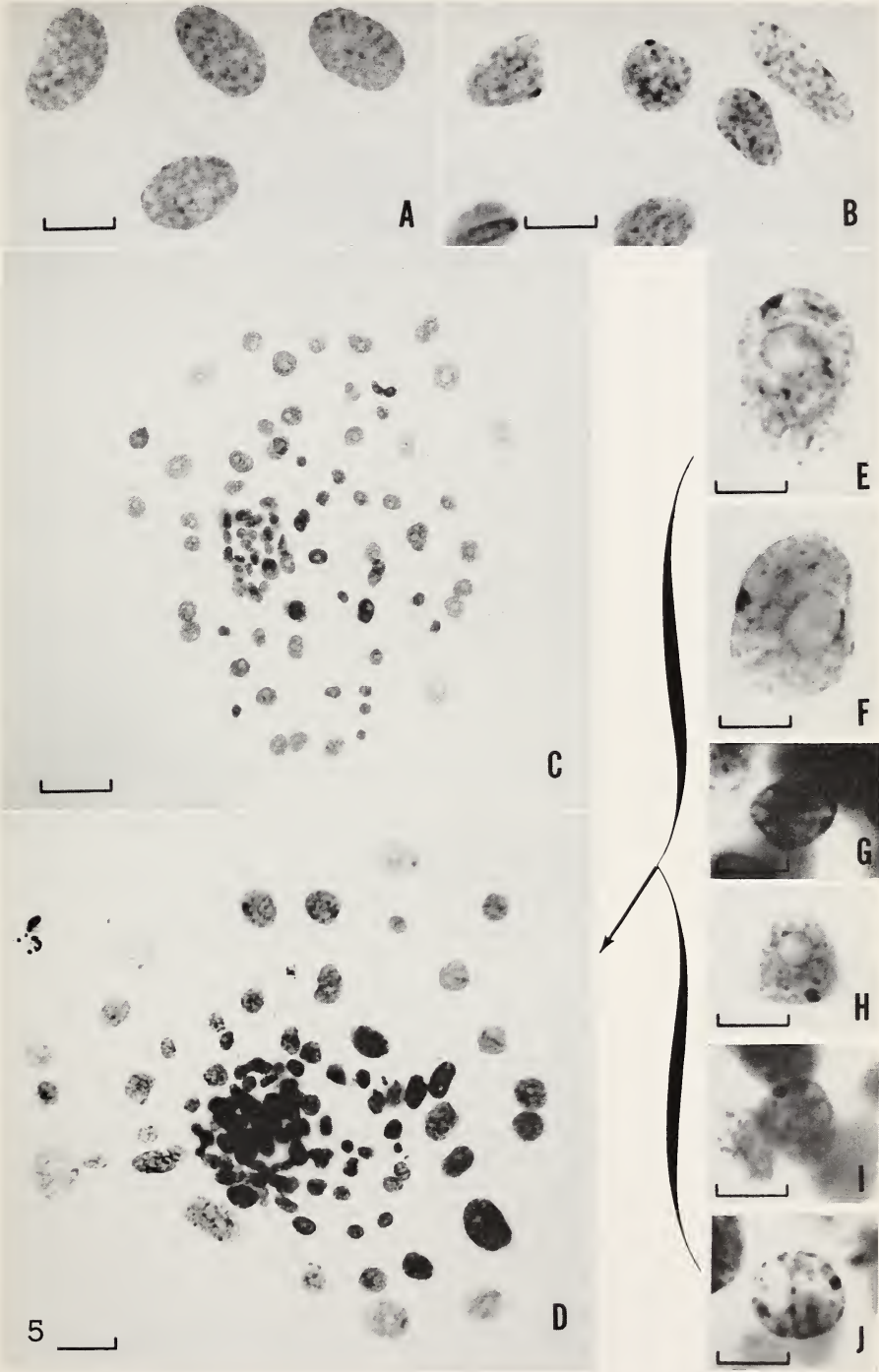


FIGURE 4A.—Metaphase of a fibroblast cultured from a human heterozygote for a normal X and an X with an abnormally large long arm. *Arrow* points to the centromere region of the chromosome so that its gross asymmetry can be appreciated. The chromosome shows the over-all decondensation characteristic of structurally normal inactive X's and it is late-replicating (unpublished observations made with Dr. Gloria Sarto).

FIGURE 4B.—Nucleus of a fibroblast stained with the Feulgen reaction, showing the characteristically bilobed sex chromatin body formed by the abnormal X described in A. A faintly visible strand of chromatin connects the lobes in the example shown, but such connections are not apparent in many nuclei.

PLATE 52

FIGURE 5.—Nuclei of cultured embryonic mouse cells stained with the Feulgen reaction. The nuclei in A and B were obtained by culturing cells from embryos of 11 days' gestation. Chromosome analysis permitted identification of A as male and B as female. C and D were "cultured" embryos (*see text*). C is presumed to be male, while D is presumed to be female. E through J are individual nuclei of embryo D. Scale markers indicate $10\ \mu$ (A, B), $25\ \mu$ (C, D), or $5\ \mu$ (E-J).



DISCUSSION

Dr. Yerganian: Dr. DeMars has continued his stimulating discussions before this group, and I believe some of our more recent observations with respect to the Armenian hamster might again open up speculation with regard to sex chromosomes—notably the X chromosome.

In this case, we have noted that distinct cell lines of monoblast origins have the distinct homologous pair of X chromosomes. Yet, recently with good euploid epithelial derivatives—notably from adenovirus-12 tumor cells—we have noted that the X chromosomes are distinctly heteroploid. In addition to this heteromorphism, there is a very prominent secondary constriction in the short arms, which in themselves are grossly differential in length.

In contrast to the lack of this nucleocyte in fibroblast explants, in order for the replication—I should say the transcription—of RNA to occur, the current concept is in relation to condensation, elongation, or diffuseness as a cytological measure of such events. For a nucleocyte to become active, there would have to be some sort of elongation—and, therefore, the possibility of the short pulse of transcription.

This is correlated with the recent observation that at this particular site there is a high degree of spontaneous reciprocal chromatin translocation, which would indicate that there would have to be stretching out of that particular strand to encourage such localized exchanges. And this is of some significance in relation to somatic crossing-over peculiar to epithelioid cells and not to fibroblasts to date.

Dr. Parshad: I wish to make some comments about the fuzzy appearance of the X chromosome described by Dr. DeMars.

For more than 10 years, we were interested in the study of chromosomes in the germ cells of a large variety of animals, *i.e.*, tiny insects, spiders, scorpions, fish, amphibians, reptiles, birds, and some of the rodents. These investigations were initiated to study the extent of chromosomal variability that can be released into the population through the germ cells. We found a great variation, both interspecific and intraspecific, in the behavior of the sex chromosomes. To give you an idea of this variation, I will take a single example which was extreme, and that was in the long-horned grasshoppers belonging to the family Tettigidae.

In most species of these grasshoppers, we found that in the earliest spermatogonial divisions the X chromosome (there is a single X in the male) behaves in the same fashion as Dr. DeMars described. That means, in the metaphase, it had a fuzzy appearance. Most of the conventional cytologists describe it as negatively heteropyknotic. But in the resting nucleus there is nothing comparable to this X chromosome.

During the last 3 or 4 spermatogonial divisions, the sex chromosome cannot be distinguished at all from the autosomes at any stage of mitosis. So, during the resting phase, we don't see any heteropyknotic body. During the prophase and metaphase, it stains and condenses to the same extent as the other chromosomes.

Then, in the premeiotic resting cell that was just going to enter mitosis, we found the nucleus had a deeply stained body, which represents the sex chromosome, and this positively heteropyknotic behavior was maintained through the prophase of the first mitotic division. During diakinesis, which is the latest period in the meiotic prophase, we found that the sex chromosome was still deeply condensed, while the autosomal bivalents had the same degree of condensation and staining as they have in metaphase. At metaphase I and during the second meiotic division, it is again indistinguishable. In the female of these species, we find the sex chromosomes (two X's) are indistinguishable in the germ cells, and they behave in the same fashion as the autosomes. Because of these observations and of the variability, I do not understand how far we are justified in relating the fuzzy appearance of the X chromosome in mammals to the one which is inactive.

Dr. DeMars: I will just quote myself. In my previous discussion, I said that the grasshopper X chromosome is often called heterochromatic and that I wasn't going to get into a discussion of heterochromatin. This is because what is called heterochromatin has so many different manifestations in different organisms and in the same organism at different times. I haven't tried to present a unified field theory of heterochromatin.

Dr. Parshad: In particular, we have the fuzzy appearance of the chromosomes in oocytes of a variety of vertebrates and salivary gland cells of Diptera at a stage when the chromosomes are very active. I do not understand how the fuzzy appearance indicates that it is inactive.

SPONTANEOUS MALIGNANT TRANSFORMATION

- Chairman:* Dr. George O. Gey, Finney-Howell Cancer Research Laboratory, The Johns Hopkins Medical Institutions, Baltimore, Maryland
- Speakers:* Dr. Leonard Hayflick, The Wistar Institute, Philadelphia, Pennsylvania
Dr. Katherine K. Sanford, National Cancer Institute, Bethesda, Maryland
- Panelists:* Dr. Georges Barski, Institut Gustav Roussy, Villejuif, Seine, France
Dr. George E. Foley, Children's Cancer Research Foundation, Boston, Massachusetts
Dr. Leslie Foulds, Chicago Medical School, Chicago, Illinois

Dr. Gey: Since this is a decennial conference, I find it difficult to chop a 10-year period from my laboratory experiences and progress because they go back more than four decades. Anyway, it is not my purpose to say much about them. I will merely mention a few things we have done relevant to the topic of this session and, more importantly, emphasize, as others have, the need for a higher degree of observational resolution of the dynamic events which cells undergo so that we may have a better opportunity to detect possible changes.

I realize the tremendous motivation on the part of some to apply machine methods to help interpret biological phenomena; *e.g.*, computer scanning for chromosome evaluation and "cyto" and "chemostat" methods for SCRAM¹ cell growth observation on growth of suspended cells (*e.g.*, MB III deBruyn-Gey). I wish these folks well in their pursuits. However, in the next decade, I believe we should do everything we can to teach the biochemists and others a little more about the habits of cells, especially at the level of dynamic morphology, and something about the cells' response to various experiences; for example, whether they are moving rapidly or slowly, show evidence of aloofness or of integration, and the variation in response of individual members. The higher resolution and detection methods must be emphasized since they are very important. Those labeling portions of chromosomes should, if possible, know the cytogenetic developmental history of the specific cells examined and also the possible future habits to be expected of the cells they are examining.

Both principal speakers have had a lot to do with what we are going to talk about and have written a great deal on the subject. They have spent much effort summarizing their own and others' experiences.

In looking at the general label covering many of our topics, we see the designation, "*in vitro*" or "*in glass*." I have often wondered whether many of you had reflected on the stupidity of many people, including our own group, in insisting that cells *must live on glass*, usually the transparent alkaline silicon oxide whose composition because of contaminants varies from manufacturer to manufacturer, and the surface of which depends much on cleaning methods. Has it a counterpart *in vivo*? It is also

¹ SCRAM—My designation of our original "suspended cells in robotized agitated medium."

important to consider whether the cells are grown deep down in the medium or up near the gas interphase where the waves, according to one of our friends from Japan, seem to wash to and fro and provide lots of opportunity for severe sheering forces to act upon them. I am sure Dr. Katsuta will tell us a little about cell experiences living in his *Nagisa* zone. Yet, I notice from his publications and my personal interviews, that these particular cells that have been greatly altered by their exposure to the gas and fluid interphase have not been demonstrated to be malignant. Our original experiences (1938-39) with cultured normal rat cells which must have received in roller tubes many of these experiences did demonstrate that some of them transformed to tumorous cells. Others that we still have growing became severely altered with great chromosomal changes but are still not tumor-producing in isologous hosts. The important point I believe is that we should not stick primarily to this glass existence for our cultured cells. We should proceed with this, of course, as far as we can, but, more importantly, we should look retroactively into their original host environment and try to understand important relationships. In our laboratory, we have approached this aspect by working extensively on the use of reconstituted tropocollagen gel as semisolid substrate and have had great success with it. I hope Dr. Foulds will attract us back into some clinical problems relating to possible progressions from normal to malignant states, and that he will help us bridge the gap between the cell's host existence and our command "*in vitro*" performances. In our own experiences with many samples of clinical material, *i.e.*, normal cervical epithelium or disturbed states, we have not yet seen a permanent alteration in the direction of malignant change. I am sure Dr. Sanford will say something about material that she has sampled from mice.

Most persons who have worked with connective tissue materials have done so because these materials are easier to work with. When we investigate cells from what is called carcinoma *in situ*, we again often find the cells' growth potential much like normal cells. There is much argument about their changed karyology and the characteristics of an idiogram constructed from them. However, when one investigates invasive carcinoma, one has a totally different picture, for here the cells often take off with vigorous growth in culture.

In connection with all of this, one cannot emphasize too much the need for great care in the selection of minute biopsy specimens whose histological composition can be evaluated as starting material for cultures. This applies also to normal tissue.

Oncogenesis *In Vitro*^{1, 2}

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SUMMARY

Spontaneous malignant transformation (alteration) is the acquisition of oncogenic properties by cultured animal cells. Its etiology is unknown. A central problem is the acceptability of criteria determining the malignant state. A vital prerequisite to the study of acquired malignant properties *in vitro* is the prior demonstration that such cells were unaltered. Other than the cells of the laboratory mouse, claims that the cells of all or most animal species frequently alter spontaneously *in vitro* are unjustified. Nonmurine cells *in vitro* commonly retain the major properties of the tissue of origin. Thus, unaltered cells *in vitro* (cell strains) retain the karyotype of the tissue of origin, multiply for a variable but finite period, do not grow in suspension culture, and do not behave like cancer cells when inoculated *in vivo*. On the other hand, most spontaneously altered cell populations (cell lines) have opposite properties and, furthermore, their behavior is often analogous to that

of transplantable tumors. One possible cause of spontaneous alterations is postulated to depend on the intervention of a virus. The suggestion is made that spontaneously altered cells, which do not differ significantly from cells altered by viruses, are induced by the integration of part of the heretofore undetected virus genome. The virus may orchestrate the whole process or it may trigger a single step. Clearly, it is important to recognize that in spontaneously altered cells the persistence of hypothetical viral genes may not be essential to maintain the altered state. Conceivably, the alteration is the consequence of transient viral action and persistent viral genes may be nonfunctional imprints. Thus, in spontaneous, as with viral alteration, a fundamental question is whether the continued expression of malignancy by the progeny of altered cells depends on the continued presence of the original inducing genome.—Nat Cancer Inst Monogr 26: 355–385, 1967.

¹ Presented at the Second Decennial Review Conference on Cell Tissue and Organ Culture, Bedford, Penna., September 11–15, 1966.

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IN THE decade since the first Tissue Culture Association Decennial Review Conference, there has been an increasingly popular acceptance of cell culture as a separate scientific discipline. We find cell culturists cast as characters in television programs, movies, and novels with such titles as "The Clone" (1) and "The Diploids" (2). Graduate students take entire one-semester courses in cell culture and even do Ph.D. dissertations on the subject. In contrast to the situation in 1956, we now even find cell culturists with good salaries. Yet I was surprised when I learned that this 4-day conference was to consider "the relationship between the cultured cell or tissue and its *in vivo* progenitor." I would have thought that the TV script writer, novelist, graduate student, and high-salaried cell culturist were all interested because it was intuitively obvious that cultured cells bear a relationship to their *in vivo* ancestors. Certainly, there doesn't seem to be any compelling evidence to the contrary. Even the phenomenon of oncogenesis *in vivo* has its *in vitro* counterpart and I have been asked to discuss this subject.

On Definitions

"I don't know what you mean by 'glory,'" Alice said. Humpty Dumpty smiled contemptuously. "Of course you don't—till I tell you. I meant 'there's a nice knockdown argument for you!'"

"But 'glory' doesn't mean a nice knockdown argument,'" Alice objected.

"When I use a word," Humpty Dumpty said, in rather a scornful tone, "it means just what I choose it to mean—neither more nor less."

"The question is," said Alice, "whether you can make words mean so many different things."

"The question is," said Humpty Dumpty, "which is to be master—that's all."

Alice was too much puzzled to say anything; so after a minute Humpty Dumpty began again. "They've a temper, some of them—particularly verbs: they're the proudest—adjectives you can do anything with, but not verbs—however, I can manage the whole lot of them! Impenetrability! That's what I say!"

"Would you tell me please," said Alice, "what that means?"

"Now you talk like a reasonable child," said Humpty Dumpty, looking very much pleased.

Lewis Carroll, "Through the Looking Glass"

The title for this session of the program, "Spontaneous Malignant Transformation," is, in itself, of interest and necessitates some discussion. The word "spontaneous" was chosen, I presume, to rule out any discussion of viral oncogenesis *in vitro*. More precisely, however, "spontaneous" means only that we are ignorant of the cause. It follows, therefore, that, if the cause of any malignant transformation *in vitro* is known, then it is ruled out of consideration here! If the limitation that the phenomenon

to be considered must be "spontaneous" puzzles us, then agreement on what is meant by the second qualification, "malignant," will certainly get all of us into a great deal of trouble. Many investigators will accept cells as being malignant only when, on introduction into the proper host animal, the cells multiply and give rise to tumors that grow progressively, are transplantable, invade and destroy host tissue, and ultimately spread and cause the death of the host. Such a demonstration has been made infrequently with animal cells, shown first not to have such properties *in vitro*. If I were to limit my remarks to cells qualifying as malignant by that definition, I should have been allowed, not 40 minutes to speak, but 4.

Obviously, this definition precludes any demonstration of the acquisition of oncogenic properties by normal *human* cells grown *in vitro*. We simply cannot make such tests in man. To complicate matters, viable human tumor tissue known to fulfill this strict definition of "malignant," often fails to grow, even when transplanted to a new site in the isogenic host. A more strict test for malignancy could probably not be imagined; yet the results with frankly malignant tissue are often negative. The implantation of cell populations into xenogeneic hosts has not resolved any fundamental problems but has made apparent the importance of bioassays for malignancy. Even limited growth of implanted cells results in a questionable conclusion. The transplantation of embryonic rudiments into older syngeneic hosts results in large growths with bizarre structures. Such embryomas may ultimately regress and are not invasive (3). Thus malignancy can be reliably deduced only if the transplanted cells grow enough to kill the host. Perhaps the medical dictionary's definition of malignant would be the most appropriate: "tending to go from bad to worse" (4). Possibly any further discussion of this subject would be malignant! Finally, a phenomenon designated "transformation" becomes clouded in the minds of those of us who are trained as bacteriologists. We understand transformation to mean the acquisition of new genetic characteristics by microorganisms exposed to the DNA extracted from another microbial population. Possibly the identical phenomenon will be observed some day with animal cell cultures, and consequently, we should be cautious not to give yet another meaning to a word already having a well-established meaning in microbiology. In view of these considerations, I have concluded that I am to address you on a phenomenon described in terms with at least two different meanings, which by definition can never be shown to occur in man, and whose cause cannot be determined!

Accordingly, I will discuss the acquisition of cancerous properties by normal cells grown *in vitro* and will call this an "alteration." An alteration presupposes the possibility of growth of cells lacking cancerous or malignant properties *in vitro* to show that such cells or their progeny can acquire these new properties. Strangely enough, this simple fact has not always been appreciated. Until relatively recently, it was generally ac-

cepted that shortly after cells are released from primary tissue and grown *in vitro* they almost always acquire abnormal or cancer-like properties.

We and others have established beyond reasonable doubt that the cells of human and other animal tissue can multiply *in vitro* for many generations before acquiring properties unlike those of the cells composing the tissue of origin (5-8). The erroneous supposition that *all* cells acquire abnormal properties after replication *in vitro* has not only led to some faulty generalizations in biology (6, 9) but to the establishment of legal requirements prohibiting the use of anything other than primary cell cultures for human virus vaccine production (10).

Let us consider briefly the mystique of the primary culture. Primary cultures are generally agreed to be composed of cell populations derived from intact animal tissue and grown *in vitro* without undergoing any subcultivations (11). The fear of subcultivations is based on the belief that such manipulations endow cells with bizarre properties precluding their use as substrates for preparation of human virus vaccines. This point is not only important in the practical area of vaccine preparation but is relevant to our discussion here. Consider a pair of sister primary cultures, one initiated with one thousand cells and the other initiated with one million cells. Suppose each culture vessel can hold a confluent cell sheet containing two million cells. It can easily be determined that when confluency is reached the culture initiated with fewer cells will have undergone 11 doublings and the other only 1. Both qualify as primary cultures and are thus acceptable substrates for vaccine production. If the culture undergoing 1 doubling is subcultivated at a 1:2 ratio 11 times, its use is forbidden, yet it has undergone the same number of total-cell doublings as the sister "primary" culture. The astute vaccine manufacturer can circumvent this regulation and at least double his yield of primary cultures by halving his inoculation density. Thus this rule may serve to encourage the very risk it was designed to prevent.

I am impressed by the observation that seldom has so much interest been evoked in a biological phenomenon as ill-defined as the one we are considering here. To discuss oncogenesis *in vitro*, I must, therefore, tell you what I understand it to be. Six years ago, we attempted to define this phenomenon and, with some minor modifications, I believe our definitions are still tenable (5). At the time, we proposed to distinguish between altered and unaltered cell populations by referring to the former as a cell "line" and the latter as a cell "strain." There was precedent for this choice in the earlier cell culture literature, but subsequent widespread use of the two words synonymously has obscured our original distinction. As we had hoped, however, the Tissue Culture Association recognized this important problem and has now sanctioned the definition of certain terms commonly used in the cell culture literature (11).

As indicated previously, one cannot have a cell alteration *in vitro* without first having unaltered cells. As simple as this may seem, numerous

investigators have reported on their ability to confer malignant properties on cell populations already known to have such characteristics. Our definitions then start with a description of unaltered or untransformed cells in tissue culture. Essentially, such cells have properties usually ascribable to the normal cells of the animal species from which they were obtained. Thus unaltered or untransformed cells originally referred to by us as composing a cell strain have most of the following five major properties:

1. Cells composing an unaltered population have the karyotype(s) of the *tissue of origin*. Thus unaltered cells of certain origins (*e.g.*, Down's syndrome) may not conform to the classic diploid karyotype. However, unaltered cells derived from *normal* human tissues not only have 46 chromosomes, but the form and shape of the chromosomes are identical with the normal human karyotype. Most female cells retain sex chromatin at interphase.
2. Unaltered or untransformed cells of all animal species have a finite potential for division in tissue culture. For example, we ascertained that normal human embryonic lung fibroblasts undergo approximately 50 cell doublings, and normal adult human lung fibroblasts undergo approximately 20 doublings (5, 6). We postulated that the finite lifetime of normal animal cells *in vitro* might be related to their expected patterns of senescence or aging *in vivo* (6, 9).
3. Unaltered cells cannot divide repeatedly in agitated fluid suspension cultures.
4. Unaltered or diploid cell strains from human tissue require cystine for growth (12).
5. Unaltered cells do not behave like cancer cells when inoculated into the hamster cheek pouch, conditioned laboratory animals, or terminal human cancer patients (5, 13, 14). Lack of cell multiplication or regression of growth occurs.

Altered or transformed populations, referred to by us previously as "cell lines," have most of the following five properties distinguishing them from cell strains or unaltered cells:

1. Altered cells have modal or submodal cell karyotypes that deviate significantly from the normal configuration or from the karyotype(s) of the tissue of origin. Such deviations are commonly differences in chromosome number and form. Occasionally only differences in form are observed (15). However, barely detectable structural changes in chromosome morphology are related to profound phenotypic changes. In human chronic granulocytic leukemia, the absence of less than half the long arm of one of the four smallest acrocentric chromosomes is directly correlated with that malignancy (16). Generally, altered cells or cell lines are mixoploid (11, 17).
2. Altered cells are capable of indefinite proliferation *in vitro*. Like transplantable tumors (their apparent *in vivo* counterparts), altered cells have evolved a mechanism for escape from the inevitability of an apparent senescence, a property of normal cell behavior *in vivo* and *in vitro*.
3. Most altered cells or cell lines can divide in agitated fluid suspension culture.
4. Most altered human cell populations can replicate in a cystine-free medium if given homocystine or cystathionine precursors (12).
5. Altered cells behave generally as abnormal cells in other ways. Unlike cell strains, when inoculated in sufficiently high density, they grow as palpable tumors in the hamster cheek pouch (13). Similar results are obtained when human cell lines are inoculated subcutaneously into terminal human cancer patients. Generally such tumor masses grow and are invasive (18).

In summary, unaltered or cell strain cells have biological properties similar to their normal *in vivo* progenitors. Altered or cell line cells have abnormal biological properties often simulating the cells composing transplantable tumors. These properties are usually irreversible. Because altered cell populations exist in which malignancy has not been demonstrated, it has been claimed that such a result disproves our view that altered cell populations are neoplastic and that only neoplastic or abnormal cells can grow continuously *in vitro* (19, 20). As indicated previously, even in man where autotransplantation of cancer tissue has been made, failures frequently occur. Southam and Brunschwig (21) reported that, of 27 patients tested, only 6 had evidence of growth of their autotransplants. More important, however, is that tumors or sublines of tumors transplantable for many years often lose this property. Furthermore, the acquisition of other new properties and the loss of old properties are not uncommon in transplanted tumor tissue. Finally, loss of the capacity to be transplanted may only reflect the loss or changes in histocompatibility that may have little or nothing to do with malignancy. Consequently, what at first may appear an exception to our contention that all altered *in vitro* cell populations are abnormal or behave like transplantable tumors is, in fact, reaffirmed to the degree that even the *in vivo* anomalies are also expressed *in vitro*.

THE PHENOMENON

It is generally held that the spontaneous alteration of a particular cell type *in vitro* follows a certain predictable sequence of events. After a variable time and number of transfers from the initial culture, cell multiplication decreases, often with subsequent cell degeneration. At this time, a cluster of cells may be found that are morphologically different from those originally composing the population and have the capacity for rapid proliferation. The event is spontaneous or fortuitous. No set of conditions is known, short of use of certain oncogenic viruses, for predictable alterations *in vitro* of nonmurine cells. However, mistreated cultures are often those most likely to change. Indeed, the general admonition of "studied neglect" often produces results. It is very important to realize that the propensity for spontaneous alterations varies not only from species to species but from tissue to tissue. Although no study has been made specifically directed toward elucidating this question, it is generally conceded that animal species can be ranked according to the predisposition of their cells to alter spontaneously *in vitro*. At the top of such a list would be the laboratory mouse whose cultured cells are found to alter spontaneously more often than not. At the bottom of the list, one could place avian and equine species whose cultured cells have

rarely yielded a cell line or spontaneously altered population, as defined earlier. Between these two extremes, one could insert the hamster somewhere in the middle and human cells somewhere at the bottom. The frequency of spontaneous alterations of human cells is very low and, with pure cultures of human fibroblasts, virtually unknown. Indeed, all altered human cells (cell lines) are epithelial in their final morphology. To my knowledge, no fibroblast-like human cell lines are known.

It may be of interest that the striking predilection for alteration of mouse cells *in vitro* may somehow be related to the propensity of the strains of animals used to develop tumors *in vivo*. Would such a high rate of alteration be encountered in cells cultured from wild mice? Does the natural incidence of tumor development in the laboratory mouse correlate with the capability of their cultured cells to alter *in vitro*? Furthermore, does the natural incidence of malignancies in the intact tissues of other animals correlate at all with the frequency of spontaneous alterations of that tissue *in vitro*?

Since the alteration of human cells is random, we were fortunate 8 years ago to see and photograph such a spontaneous alteration in a culture of human amnion cells. I believe this was the first photograph of a spontaneous alteration in human amnion cells where the altered and unaltered cells were seen side by side (22) (fig. 1). The culture was 35 days old when a number of foci of altered cells were found. The culture had previously undergone three subcultivations and the growth of the unaltered cells had diminished considerably when six foci of altered cells were discovered. There is a subjective impression from the literature that spontaneous alterations of human cells occur more frequently in amnion than in any other tissue. Such a conclusion is questionable since amnion is, perhaps, the most readily available normal human tissue for experimental study. If similar quantities of epithelial cells from other human organs could be obtained with comparable ease, this impression might be invalidated. However, the amnion cells depicted here were those obtained from my daughter at her birth and, for what it is worth statistically, amnion cells grown from my other four children have yielded negative results. The spontaneously altered amnion was derived from my daughter Susan and named WISH (Wistar Institute Susan Hayflick), according to the recommendations for naming cell lines established in 1957 at the International Tissue Culture Meeting in Glasgow, Scotland. WISH has been in continuous cultivation for 8 years. It is mixoploid, grows in suspension, and forms palpable nodules when inoculated in sufficient number into the hamster cheek pouch. It is, therefore, a spontaneously transformed or altered cell population, but are the cells malignant? If only the strictest criteria for demonstrating malignancy are requisite, then I can tell you that Susan has wisely refused to permit the inoculation of WISH into her forearm.

There is certainly no doubt that the conversion of normal animal cells grown *in vitro* to cells having malignant properties is a *fait accompli*. It is also generally accepted that this phenomenon can occur fortuitously and without apparent cause. The central issue, however, devolves around criteria for demonstrating the event and an explanation of the mechanism by which it occurs. I do not intend to belabor the point of acceptable criteria for showing the oncogenic properties of altered cells. The literature is now replete with perfectly acceptable demonstrations of the acquisition of oncogenic properties by cultured normal cells either occurring spontaneously or virus-induced. The most difficult question, that of the possible mechanisms, remains.

ON MECHANISMS

As considered earlier, malignant alterations when qualified by the adjective "spontaneous" place us on the horns of a dilemma, since it set for us the task of explaining an event that by definition must have no known cause! We must, therefore, escape the dilemma by slipping between the horns and comparing oncogenesis *in vitro* of unknown cause with oncogenesis *in vitro* of known cause.

Many cell properties change because of cellular alteration and some have been used as an index for recognition of the phenomenon; for example, changes in karyotype, morphology, growth properties, or metabolic patterns. Taken separately, these parameters cannot be used as a measurement of carcinogenesis *in vitro* or *in vivo*. As already indicated, the only indubitable proof is demonstration of progressive growth leading to death of the organism when the cells in question are implanted in the appropriate host. Failure to grow under such circumstances does not, by itself, mean that the cells are "normal," but a positive result is unambiguous.

Contact Inhibition

The behavior of cells in tissue culture, known as contact inhibition, has often been regarded as a parameter by which altered cells may be distinguished from unaltered. Consequently, contact inhibition is of considerable interest if it is an expression of the invasiveness of a malignant cell. The cell surface is a distinctive organelle in the hierarchy of tissue organization, so normal cell behavior may well be contact-dependent. Likewise, cell surface changes appear to be significant in spontaneous and virus-induced alterations *in vivo*. The phenomenon of contact inhibition is described as the "stopping of locomotion in a particular direction by contact with another cell" (23). In recent years virologists have observed, as a result of the introduction of oncogenic viruses, foci of

piled-up cells in the monolayer bed. This has often been thought to occur because contact inhibition not only establishes the pattern of monolayer sheet formation but also effects a concomitant switching off of mitotic activity.

Thus oncogenic viruses somehow interfere with the switch, and the infected cells go on dividing as foci of piled-up cells. The interpretation that contact inhibition necessarily influences mitotic activity is unproved. Indeed, it is very unlikely that as a result of the "stopping of locomotion in a particular direction by contact with another cell" mitosis stops in unaltered cells. Certainly in lightly seeded cultures, impinging cells do not cease to divide, but continue to form a monolayer. The degree of monolayering, which perhaps reflects the efficiency of contact inhibition, is subject to many different environmental influences. This is even manifest in cells infected with an oncogenic virus in which the medium components and cell population density directly effect the multilayering or piling-up (24). Secondly, the production of collagen or other intercellular matrix material can, by preventing intimate cell membrane contacts, circumvent the expression of contact inhibition (23). We showed some 5 years ago that human diploid cell strains, which are normal cells by any reasonable criteria, when left unfed for a few weeks and with only intermittent adjustments of pH can produce membranes composed of polarized fibroblasts growing in two or more parallel planes (5) (figs. 2 and 3). Normal cells are also arranged three-dimensionally *in vivo*; thus cessation of mitosis is not a necessary consequence of monolayer formation. The significant point here is that mitotic activity, *per se*, has not been shown to shut off as a result of contact inhibition. Furthermore, probably the piling up of cells is not as significant as the *way* in which they pile up. Multilayered membranes occur with unaltered cells as a result of the process of a highly ordered pattern of fibroblast behavior, usually with the cells oriented in parallel. With spontaneous and virus-induced altered cells, the piling up is usually made manifest by a disordered, random, cellular array. By use of a perfused T-60 flask, which ordinarily holds about 4×10^6 cells in a monolayer, 25×10^6 human diploid cells can be grown (25). Such huge cell volumes result in ordered multilayer formation in human diploid cells with normal fibroblast properties.

Suspension Cultures

Of possible related interest is the observation that unaltered cells are incapable of sustained multiplication in suspended culture under identical conditions in which altered cells proliferate (5). It is unreasonable to suppose that, for example, the multiplication of human diploid cells in suspension reflects inadequacies in the medium, for the same medium that supports their luxuriant growth in monolayers fails to allow them to proliferate in suspension. Obviously, the explanation must lie with prop-

erties of the physical environment. Unaltered cells must require contact of their membranes with solid surfaces in order to replicate. It would be interesting to increase the viscosity of fluid medium with, *e.g.*, methylcellulose, to determine at what point adequate "surface" conditions allow for cell multiplication. This behavior of unaltered cells may be, like contact inhibition, another manifestation of the orchestration of cell deportment by the contiguity of the limiting membrane with surfaces.

Some Properties of Altered Cells

Some recent observations are important here, since they have shown that the acquisition of malignant properties by cultured mouse cells is not abrupt but gradual. Furthermore, when the first mixoploid cells emerge in the population, tumors are only occasionally produced and host survival time is long. The incidence of tumor production appears to increase as the cell doublings accumulate *in vitro* with C3H mouse tissue (7). Others have also observed the same phenomenon in the cells of other strains of mice (19, 26). Mixoploidy then does not *ipso facto* coincide with demonstrable oncogenicity. However, one must be wary in arriving at such a conclusion, for a determination of chromosome anomalies is limited by existing techniques and the observational level used. A small deficiency or rearrangement in one of the smallest human chromosomes, as observed in the light microscope, can result in profound phenotypic manifestations (16). Chromosome changes taking place at levels below our present capabilities of detection (small deletions or point mutations), of course, cannot be ruled out as the primary cause of malignant alterations. The Boveri theory is still very much intact.

As also demonstrated, malignant alterations can occur in cell populations with or without gross morphological changes. The event has occurred in populations subcultivated with enzyme preparations or by mechanical dispersion and in totally isologous environments (27) as well as in numerous kinds of media supplemented with heterologous animal sera or tissue extracts (19).

From findings made over the past decade, it seems fair to say that the acquisition of malignant properties by presumably normal cells cultured *in vitro* is not an inevitable consequence of multiplication in culture. Generalizations based on the relatively high rate of alteration of mouse cells, on which much of the early work was done, can be misleading if extended to include the cells of other species. Oncogenic alteration is as rare in some species as it is common in the cultured cells of the mouse. If the property of indefinite propagation is any guide, only as recently as 1962 have fish and insect cells been shown capable of long-term culture (28, 29). These findings established that cells other than those from mammals can undergo a spontaneous alteration *in vitro*. Surprisingly and perhaps significantly, spontaneous alterations in fowl cells, especially in

such commonly cultured tissues as those derived from chickens, are unknown or at least have never been confirmed.

In Vitro Alterations of Tumor Tissue

A superficial consideration would lead one to expect that the cultivation of frankly malignant animal cells should consistently yield cell lines with the attributes of altered cells, as defined above. That this does not happen should be emphasized. A comparable number of altered populations have been derived from both normal and malignant tissue from many animal species including man (30). In our experience in cultivating the cells of approximately 500 different human tumors, only two spontaneously altered. Curiously, the two tumors were glioblastoma multiformes (31). As already indicated, the predisposition for alteration of normal human tissue is also very low, perhaps of the order of less than 1%. Of considerable importance in postulating a reason for this apparent contradiction is that the establishment of altered populations of cancer cells *in vitro* is more frequently accomplished with the cells of tumor tissue that have been transplanted frequently *in vivo*. Consequently, it can be concluded that the changes resulting in frankly malignant cells *in vivo* do not concomitantly give to those cells all the properties of altered cells *in vitro*. Specifically, such parameters as diploidy, lack of multiplication in suspension, and inability to proliferate indefinitely are common *in vitro* properties of untransplanted primary tumor cells. The cells of transplantable tumors, however, are almost always mixoploid (heteroploid) and more easily yield altered cell populations *in vitro*, characterized by the ability to proliferate indefinitely. The analogy of the behavior of transplantable cancer cells *in vivo* with many continuously passaged altered populations *in vitro* suggests that many altered cell cultures may be the *in vitro* expression of that *in vivo* phenomenon. The low frequency with which primary cancer cells of many species adapt themselves to continuous *in vivo* passage is similar to the low frequency with which such cells make a similar adaptation *in vitro*. Equally impressive is the high correlation between *in vivo* tumor transplantability and acquisition of the properties of altered *in vitro* cells. On that basis we deduce that the acquisition of the properties of altered *in vitro* cells is not a natural consequence of the acquisition of malignant properties by primary cancer tissue. However, subsequent *in vivo* passage of cancer tissue as transplantable tumors frequently endows the cells with such properties. Thus the underlying theme of gradation or stepwise progression in the acquisition of full malignant properties is again observed (32).

It is an oversimplification to consider altered cells as the simple equivalent of "normal" cells in experiments where rigid criteria for malignancy have not been demonstrated. Such cells plainly differ from normal cells

in other parameters and may have as their counterpart, in the tissues of the intact organism, benign tumors or preneoplastic lesions.

CHERCHEZ LE VIRUS

To speculate on the cause of spontaneous malignant alterations *in vitro*, without referring to the induction of malignant alterations *in vitro* by viruses, is impossible. The two phenomena are similar and I propose that ultimately many spontaneous alterations will be found to have a viral etiology. Evidence is now sufficient for the conclusion that viruses, such as polyoma, SV40, and Rous sarcoma, can alter normal animal cells to the malignant state, even by the most rigid definition. Such viruses as these represent the only conditions known for reproducibly changing the monolayer cultures of normal cells of many animal species to the neoplastic state (figs. 4 and 5). Recent claims to have done this by chemical means have not yet been confirmed (33). The mechanism by which viruses effect this change is now under investigation in numerous laboratories and, because of the many possibilities for virus interaction with cell constituents, it augurs to be a complex area of inquiry. There is, however, a provocative conceptual analogy between the behavior of these oncogenic viruses and the lysogenic state in bacteria. For example, bacteria infected with temperate bacteriophages can undergo changes in antigenicity, colonial features, and other heritable variations (34, 35). Thus the genetic control of bacterial cell properties by phage offers an attractive model relevant to the induction of oncogenic properties in normal animal cells by viruses. Indeed the discovery of a new antigen in cultured animal cells, altered by viruses *in vitro* but no longer shedding virus, has led to pursuit of the relation of this T- or neo-antigen to all or part of the virus genome. If the temperate bacteriophage system is a significant model, it should be possible to induce the production of virus by nonproducing altered cells. Although efforts to identify the neo- or T-antigen with the virus genome have been frustrated, the search is continuing with more sophisticated tools (36, 37).

Such hypothetical integration of a viral genome with the DNA of altered cells might be recognized by techniques similar to those used for the rescue of integrated bacteriophages and certain animal viruses with a virus of the same type but differing in some genetic marker. Current data with oncogenic viruses lead to the conclusion that some virus genes, but not a complete infectious virus genome, persist in cells altered by DNA viruses. Some of these persisting genes may be involved in the synthesis of the neo- or T-antigen and others may produce the protein that changes the antigenic properties of the cell surface and is detectable by transplantation experiments (38, 39). Other genes are absent or inactive,

especially those directing the synthesis of the main constituents of the viral protein coat. The "helper virus" phenomenon in the Rous system or the SV40-adenovirus hybrid system suggests ways in which the integration of information by other oncogenic virions with their target cells may result in malignant manifestations. There should be no implication here that precise identity exists between the lysogenic state in bacteria and oncogenic viruses in mammalian cells but that virus induction of new host cell properties occurs in both cases which do not ultimately depend on the synthesis of mature virus. Resistance to superinfection is also characteristic of both systems. Thus clues to be found in the microbial system should not be overlooked, especially since the synthesis of new proteins is a frequent consequence of phage infections.

Of considerable importance to my suggestion that spontaneous malignant alterations may be induced by viruses is the recent observation by Girardi and Jensen (40). They have shown that adenovirus type 7, usually incapable of altering African green monkey kidney cells *in vitro*, can do so under certain conditions. This virus can produce a cytopathic effect in other more sensitive cells at a titer of 10^{-7} , but cannot do so in African green monkey kidney cells at a concentration of 10^{-1} . However, at other low virus concentrations where no cytopathic effect occurs, alterations of the African green monkey kidney cells do take place. This is the first reported alteration of primate cells by an adenovirus. Is it possible then that other common viruses tested at dilutions greater than those causing cytopathology will be found to induce cell alterations?

What are the similarities and differences between spontaneous malignant alterations and virus-induced malignant alterations? Do these parameters provide clues for the possible mechanism of spontaneous malignant alterations? It is now well established that normal cells altered by polyoma and SV40 develop significant karyotypic changes and that such cell populations can multiply, apparently indefinitely. Cells altered by viruses have been demonstrated to be malignant and can grow in suspension. Some viral alterations have a lytic stage comparable to that found in spontaneously altering cultures. Such cells can grow in suspension and acquire their malignant properties slowly as do cells spontaneously altered. Alteration of normal chick cells by Rous sarcoma virus, although endowing them with malignant properties, has not been reported to release them from the inevitability of loss of division potential *in vitro*. Thus chick cells, in which a spontaneous malignant alteration has not been reported, are also apparently incapable of indefinite serial culture *in vitro*, even after infection with Rous sarcoma virus (41).

One possible characteristic distinguishing cells that have acquired malignant properties spontaneously from those acquiring such properties by viral intervention is the possession of a novel antigen in the latter. As indicated, the role of the neo-antigen is still uncertain, and indeed, its identity or relationship to the inducing virus is still a mystery. On the

other hand, novel antigens have been reported in spontaneously altered cell populations and, by analogy, may be the telltale signs of an inducing agent (42, 43).

For those of us in search of hypotheses, one of the most intellectually satisfying would be that spontaneous malignant transformations are induced by the integration within the host cell of part of a virus genome. At one extreme, the virus may orchestrate the whole process, and at the other, it may trigger a single step. The characteristics of the phenomenon that are identical in the spontaneous and virus-induced systems are certainly impressive and lead one to consider that one possible cause of the spontaneous event may be a virus.

Characteristics Shared by Cultured Cells Altered Spontaneously or by Viruses

1. Relatively few cells in the normal population alter.
2. A morphological change usually occurs.
3. Unaltered cells in the altering population usually lyse or degenerate.
4. Karyotype changes usually result first in increased tetraploidy and ultimately in mixoploidy.
5. A time lag exists between morphological changes and demonstrable tumor induction.
6. A time lag exists between karyotype changes and demonstrable tumor induction.
7. A loss of contact inhibition usually results in a random pattern of piled-up cells.
8. Most altered populations can proliferate in suspended culture.
9. Altered cells often acquire the ability to proliferate indefinitely.
10. Most altered populations can multiply when inoculated in sufficient numbers *in vivo*.

Despite the fact that known chemical carcinogens have been tested for their carcinogenic properties on *in vitro* cultivated monolayers of normal cells, no confirmation of this reaction has been forthcoming (33). The mystery of why neoplasms can be induced easily *in vivo* and not *in vitro* by known chemical carcinogens and irradiation makes it less likely that such agents cause spontaneous malignant alterations. At present, such materials appear to have a requirement for the intact organism to manifest themselves as being carcinogenic. Perhaps one other important piece of evidence in favor of the viral hypothesis of spontaneous malignant alterations is the series of alterations that have occurred in different human cell cultures grown in various laboratories and in which subsequent attempts to repeat such findings have been frustrated (44-47). If, in fact, the neo-antigens associated with cells converted by oncogenic viruses are distinctive hallmarks of virus alteration, then the search for imprints left by hypothetical viruses associated with spontaneous malignant alterations should be pursued.

Clearly it is important to recognize that in spontaneously altered cells persistence of hypothetical viral genes may not be essential in maintenance of the altered state. Conceivably the alteration is the consequence of transient viral action and persistent viral genes are nonfunctional im-

prints. Thus in spontaneous, as in viral alteration, a fundamental question is whether the continued expression of malignancy by the progeny of altered cells depends on the continued presence of the original inducing genome.

A model for this hypothetical cause of spontaneous malignant alteration *in vitro* may be the recent findings with Burkitt's lymphoma cells. These cells, unlike those of other human tissue, establish themselves with apparent ease as continuously propagable populations, and a number of these cell lines are now known (48-51). Although the etiologic agent of this disease has not been isolated, a virus is strongly implicated. Very recently, serological data and visualization of herpes-virus-like structures in the altered cell populations obtained from lymphomatous tissue have produced circumstantial evidence for the presence of a virus (51, 52).

Perhaps this approach to the problem will show that the spontaneity of malignant alterations *in vitro* ultimately will be ascribable to the insertion of new genetic information by viruses into the normal host cell. Such viruses, behaving as migratory components of the cell genome, may act on the host by controlling new syntheses or exerting new regulatory functions which, in turn, are regulated by the cellular milieu. Since viruses contain the elements of genetic material that are not fundamentally different from those of the cell, it is reasonable to suppose that viruses can be instrumental in cell growth regulation and differentiation just as can other classes of genetic changes. Specifically, in the induction of oncogenic changes, such viruses may have somewhat less integrated or more "foreign" genetic material leading to interference with the normal regulatory mechanisms of the host cell.

Viruses offer unique opportunities for analyses of the integrated patterns between two genetic systems. Even though their role as etiologic agents of spontaneous alterations may ultimately prove rare or even nonexistent, they still deserve the place they have recently assumed in the center of the stage of cancer research.

In conclusion, the presence of viruses in mediating what would be called here spontaneous malignant alterations *in vitro* is one of the most attractive hypothetical mechanisms for its genesis. We might all wonder, with good reason, why more convincing evidence for this hypothesis has not as yet been provided. To that I will say, as do the highway construction signs, "We are sorry, please be patient and bear with us during this temporary inconvenience."

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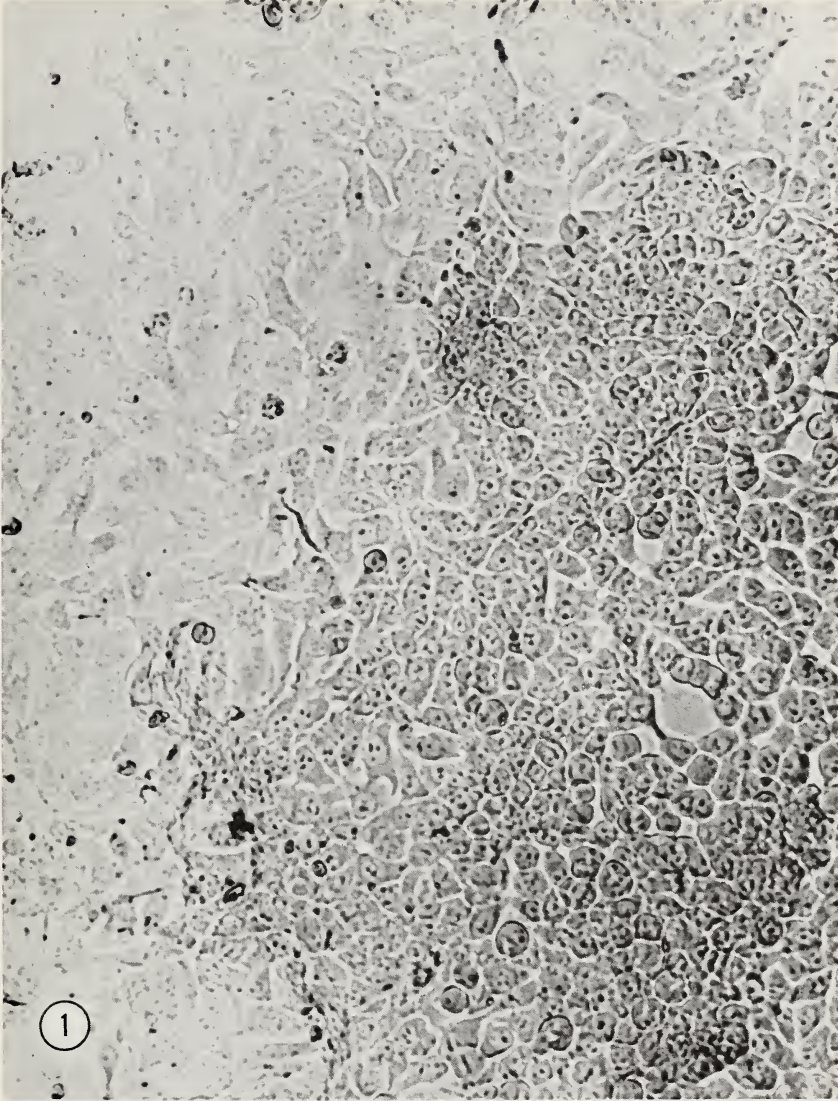


FIGURE 1.—Spontaneous alteration occurring in a 35-day-old culture of human amnion cells. Altered cells in the island *on right* are morphologically distinguishable from the unaltered population *on left*. Mitotic activity, apparent in the altered cells (cell line), is lacking in the unaltered population, which has a finite lifetime *in vitro*. The resulting cell line consisting of mixoploid cells is called WISH and has been in serial cultivation for 8 years. Bright-field photograph.

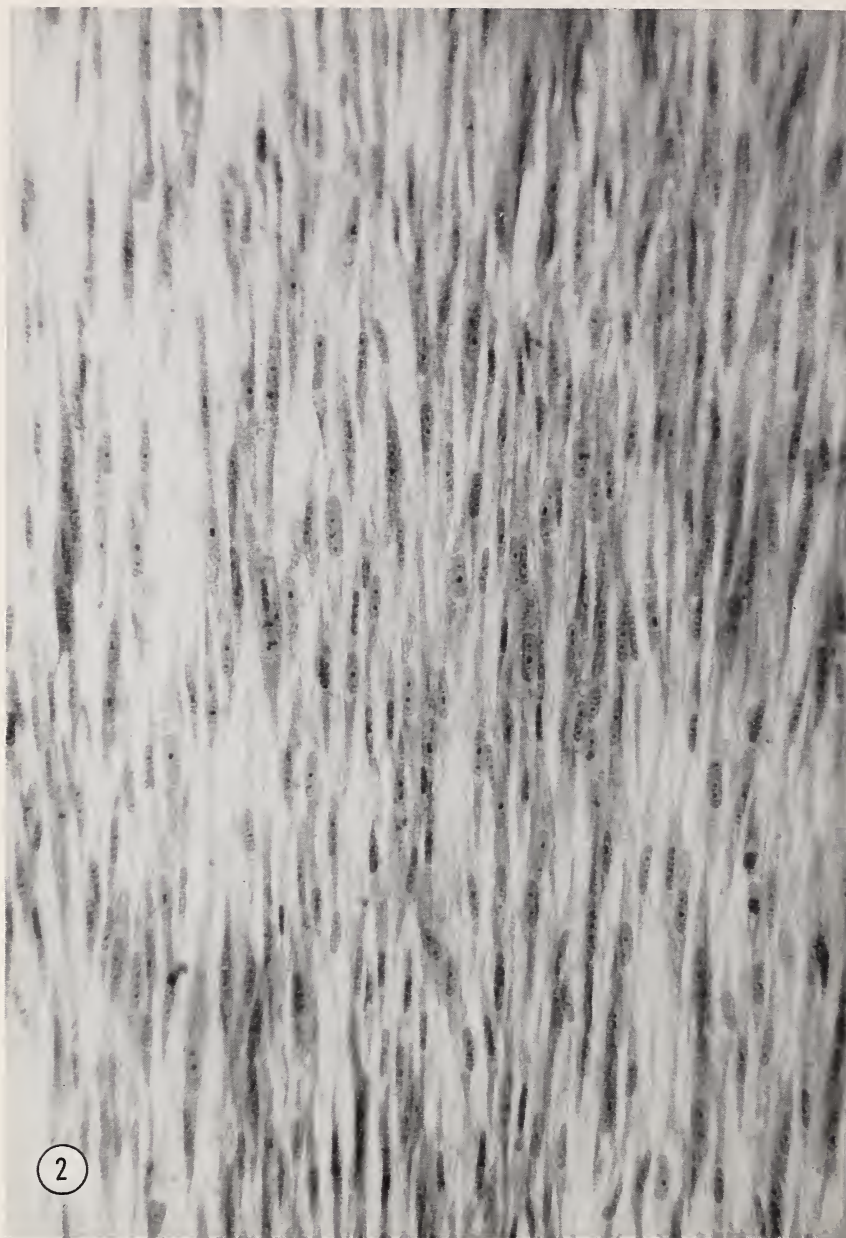


FIGURE 2.—Unaltered human diploid cell strain WI-38 in confluent monolayer growth after 3 days in culture. Highly polarized unidirectional cell orientation is presumably the result of contact inhibition. May-Grünwald-Giemsa.



FIGURE 3.—Multilayer growth of WI-38 after incubation of the unfed culture at 37 C for 3 weeks. At least two planes of cell growth are visible, each consisting of fibroblasts in a polarized orientation. Ultimately, the membrane dislodges from the glass surface and consists of cell layers separated by a collagenous matrix (5). May-Grünwald-Giemsa.

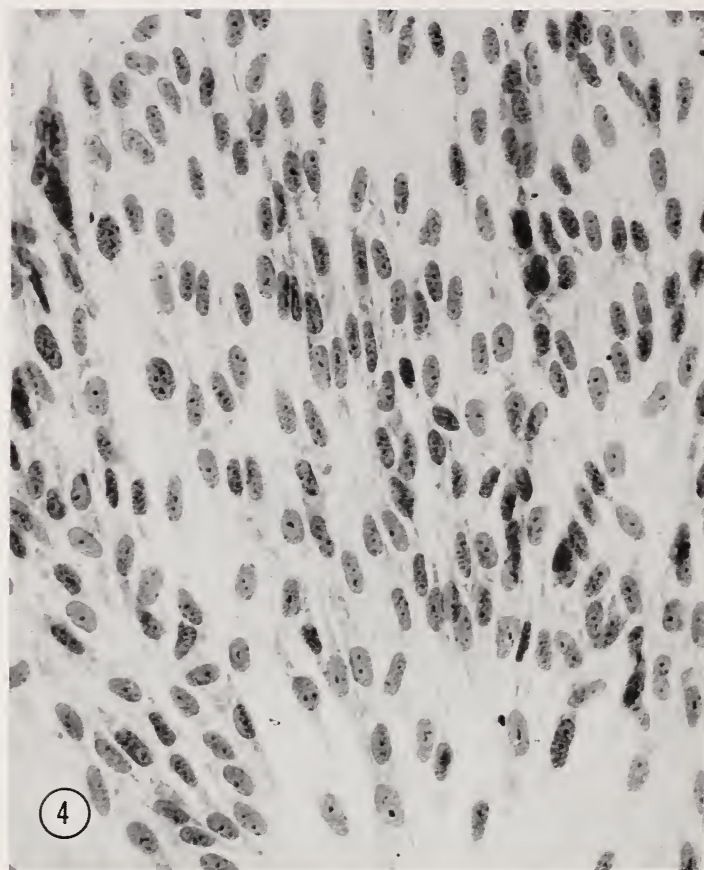


FIGURE 4.—Monolayer culture of the unaltered, human diploid cell strain population WI-38. This strain was derived from the lung tissue of a 3-month-old female embryo. Sex chromatin is apparent at the rim of many nuclei. May-Grünwald-Giemsa.

Photograph courtesy of MR. F. C. JENSEN

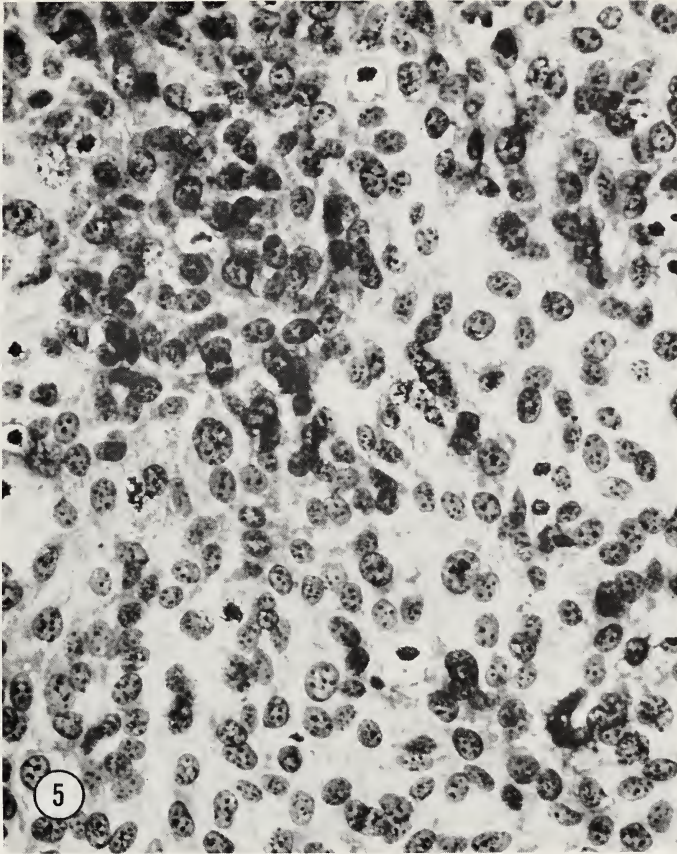


FIGURE 5.—WI-38 altered by the oncogenic simian virus #40. Compare with figure 4. *Note:* 1) great mitotic activity even after confluency, 2) random piling up of cells, 3) greater proportion of nuclear mass to cytoplasm, 4) larger average number of nucleoli per nucleus. May-Grünwald.

Photograph courtesy of MR. F. C. JENSEN

DISCUSSION

Dr. Barski: I was told that Queen Elizabeth I of England on her death bed rose suddenly and shouted out, "What is the answer?" Then she went into a coma, but rose again, for the last time, and shouted out, "What is the question?" I think Dr. Hayflick was right to devote the introduction of his speech to the question, which is our question today: What is the meaning of "spontaneous malignant transformation" and, especially, the trouble with the definition of malignancy itself.

As a matter of fact, the result of the most simple test of malignancy which is inoculation of cells into an appropriate recipient animal will depend on many things—some of them very simple and some quite complex. We know there is a threshold dose of cells necessary to produce a tumor. If we are below this dose, the cells will be rejected by a mechanism that is not very clear. But this may be a general homeostatic mechanism by which a cell, malignant or normal, when introduced into an organism, will be as a rule rejected if it is not there in the right place, unless this mechanism is surmounted.

Actually, I think we will avoid many difficulties if we accept that malignancy is not an all-or-none phenomenon. This could be a revelation for some people, but certainly not for those who are observing daily clinical or experimental cancer. We have to bear in mind that cells *in vivo* and *in vitro* can be less or more malignant, less or more invasive. Therefore, when we accept the notion of a range of malignancy, we can easily on this range imagine point zero, no malignancy. This is directly linked with the problem of malignant transformation *in vitro*. The cells transformed *in vitro*, as Dr. Sanford emphasized, present usually a wide range of malignancy, and in some cases can be transformed and not malignant at all.

I would also like to point out that it is a good thing to realize the capacity of transformed cell lines to produce tumors depends on a set of characteristics. It may be necessary for many of these characteristics to coincide to produce a tumor.

These single characteristics are, for instance, the incapacity to respond properly to still obscure but certainly existing integrating or morphogenetic central mechanisms, adaptation to host-provided nutritive conditions, incitation of local vascular reaction, and, then, essentially, the acquisition of a positive property to invade the surrounding normal tissues, which depends on many things like cell movement, decrease of cohesion between cells, capacity to crush normal cellular and intercellular structures, and, finally, capacity to withstand the immunological reaction of the host.

The essential question, which was raised and discussed to some extent in a controversial way by the two speakers, is: What is the relation between transformation or alteration of normal cells in long-range cultures and malignant transformation of these cells? We believe, according to data obtained in our laboratory, that certainly these two phenomena, alteration and malignant transformation, are not equivalent.

According to our experience with C57BL adult mouse lung tissue, following nine different and successful attempts to establish cell lines from this tissue starting from the 4th month after the establishment *in vitro*, we observed transformation of the karyotype and then later appearance of malignancy during the first year of culture, but not for all the lines. For most, we had the malignant transformation. But at least one, the P6 line, now in more than 3 years' culture, is nonmalignant according to the standard inoculation tests in newborn or adult isologous animals.

We have no reason to believe that these negative results of animal inoculation are due to an antigenic shift of the cells with respect to the recipient animal. We wish to emphasize that the highly malignant cell strains showed morphologically a very particular intermingling and random and overlapping type of growth and single cells of the malignant lines were more independent and perhaps more rapid in their proper movements. There is also less cohesion, as Dr. Sanford suggested, between cells of the malignant type.

However, in the case of lung tissue cells, we can say that we have, starting from the beginning of the culture, a mixed cell population. One can imagine easily that some of these cells having their proper morphology are more prone to malignant transformation than others. The answer to this question would be to start with cellular clones obtained from primary cultures.

This sounds like a reasonable program for the next 10 years to see whether all cells present originally in a normal tissue are equally capable to transform and to produce eventually malignant growth.

Finally, I wanted to emphasize that what we know about the cell transformation problem is limited to only a few species—chiefly, mice, rats, golden and Chinese hamsters, and, to some extent, man. We started recently a study of the cell lines from guinea pig lungs and noticed already that things are occurring quite differently with this species than in human or mouse cultures.

So far, after 2 years of culture, attempts to produce tumors with the *in vitro*-adapted guinea pig cell lines failed according to the results obtained by inoculation of inbred and syngeneic guinea pigs.

But cell transformation experiments using cells from different species appear as another good program for the next 10 years.

Dr. Gey: Thank you very much, Dr. Barski. You brought up issues relating to heterosis and degree of mosaicism that exist in the original tissue and the need to uncover the potential of that tissue.

Would Dr. Hayflick care to say something about the issues of rapid versus slow transformation? You mentioned that in your talk, and it was again brought up by Dr. Barski. Have you any comments you would like to make?

Dr. Hayflick: No.

Dr. Gey: Dr. Sanford?

Dr. Sanford: I was interested in Dr. Barski's work with the guinea pig tissues. I wondered if he tried any tissues giving rise to fibroblast cells. I think they may be more likely to undergo this change.

Dr. Barski: Actually, the morphology of the cells from guinea pig lung tissue was rather fibroblastic, though there were some differences between parallel lines. I would like to add that after 6 months of *in vitro* growth in one case, we could, with Miss Cornefert at The Wistar Institute, rescue the cells that were stagnated by infection with the SV40 and grow them for another 6 months, but they did not show any complement-fixing antigen of the SV40 type. However, a parallel line originating from another guinea pig lung could be cultivated *in vitro* for 16 months without any known viral infection.

Dr. Foley: It seems to me that there have been two significant advances in this area of experimental biology since the last Decennial Conference: (a) the demonstrated neoplastic transformation of normal cells by virus, and (b) more or less general agreement with the concept that not all cells in culture are neoplastic, and that heteroploidy may develop *in vitro* for other reasons and should not in itself be equated with neoplastic transformation. The evidence reviewed by Dr. Sanford and Dr. Hayflick appears to be in general agreement on both these points.

Some years ago, when the ideas embodied in the latter concept were not always viewed as entirely respectable, we (for reasons not germane to the present discussion) became interested in distinguishing between cell lines derived from normal as opposed to neoplastic tissue, if indeed such distinction were possible. Lacking any other kind of marker, we resorted to comparison of the biological behavior of cells in culture on transplantation to an experimental host. Any definition of experimental malignancy is in itself tenuous at best, and such definition becomes particularly tenuous if one is interested in the biological behavior of human cells, as we were at that time; thus this kind of experiment was limited to the use of a heterologous host.

The cheek pouch of the Syrian hamster was used in these experiments, primarily because of the background data already accumulated on the acceptance of heterologously transplanted neoplastic tissue taken directly from the patient and the rejection of similarly derived normal or benign tissue when heterologously transplanted to the cheek pouch of the Syrian hamster. These studies,¹⁻⁸ which included more than 40 established cell lines derived from normal, neoplastic, and embryonal human tissue, are summarized in table 1. All these cell lines were established in culture, and all were heteroploid. Their behavior on heterologous transplantation to the cheek pouch of the Syrian hamster, with one or two exceptions, correlated with the origin of the cell line; those derived from human neoplastic tissue survived and grew in the cheek pouch, whereas those derived from human non-neoplastic tissue failed to survive and grow in the cheek pouch, being promptly rejected by the hamster.

The results obtained with cell lines derived from human embryonal tissue are of particular interest, in that some of these behaved like those derived from adult human non-neoplastic tissue, whereas others survived and grew in the cheek pouch, and for practical purposes were indistinguishable in this heterotransplantation system from those cell lines derived from human neoplastic tissue. In each instance, classification of the behavior of these cell lines was based on histologic examination of the implanted cheek pouches, gross evaluation of the appearance or persistence of a nodule at the site of implantation being insufficient basis for distinction. For example, the histology of a nodule resulting from the implantation of a subline⁹ of the KB cell¹⁰ is illustrated in figure 1, in contrast to the histology of a nodule resulting from the similar implantation of a cell line derived from the peripheral-blood buffy coat of a normal laboratory technician,¹¹ as illustrated in figure 2. The histopathologic differences between these nodules are quite evident, figure 2 illustrating the inflammatory response to, and the rejection of cells of non-neoplastic origin by, the recipient hamster. Moreover, nodules resulting from the heterotransplantation

¹ HANDLER, A. H., and FOLEY, G. E.: Growth of human epidermoid carcinomas (strains KB and HeLa) in hamsters from tissue culture inocula. *Proc Soc Exp Biol Med* 91: 237-240, 1956.

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⁶ FOLEY, G. E., HANDLER, A. H., ADAMS, R. A., and CRAIG, J. M.: Assessment of potential malignancy of cultured cells: Further observations on the differentiation of "normal" and "neoplastic" cells maintained *in vitro* by heterotransplantation in Syrian hamsters. *Nat Cancer Inst Monogr* 7: 173-204, 1962.

⁷ FOLEY, G. E., and HANDLER, A. H.: Studies on the heterotransplantability of some diploid cell lines. In *Proceedings—Symposium on the Characterization and Uses of Human Diploid Cell Strains* (Institute of Immunology, Zagreb, eds.). Opatija, Yugoslavia, International Association of Microbiological Societies, 1963, pp 429, 447.

⁸ CASPERSSON, T., FOLEY, G. E., KILLANDER, D., and LOMAKKA, G.: Cytochemical differences between mammalian cell lines of normal and neoplastic origins. *Exp Cell Res* 32: 553-565, 1963.

⁹ FOLEY, G. E., HANDLER, A. H., LYNCH, P. M., WOLMAN, S. R., STULBERG, C. S., and EAGLE, H.: Loss of neoplastic properties *in vitro*. II. Observations on KB sublines. *Cancer Res* 25: 1254-1261, 1965.

¹⁰ EAGLE, H.: Propagation in a fluid medium of a human epidermoid carcinoma strain KB. *Proc Soc Exp Biol Med* 89: 362-364, 1955.

¹¹ BERMAN, L., STULBERG, C. S., and RUDDLE, F. H.: Human cell culture. Morphology of the Detroit strains. *Cancer Res* 17: 668-676, 1957.

TABLE 1.—Differential heterotransplantability of human cell lines in Syrian hamster cheek pouch

Source tissue	Number of cells in inoculum	Characteristic response
Normal, adult*.....	10 ⁶ –10 ⁵	(–) No evidence of active growth, necrosis, non-transplantable nodule
Neoplastic.....	10 ⁴ –10	(+) Actively growing, invasive, transplantable tumor
Embryonal*.....	May exhibit	characteristics of either normal or neoplastic cell lines

*Some of these cell lines elicit no response, even with inocula of 10⁶ cells.

of cell lines derived from human neoplastic tissue were serially transplantable in the hamster cheek pouch, whereas those nodules resulting from the implantation of cell lines derived from human non-neoplastic tissue were nonviable, as adjudged by their failure to grow when serially transplanted.

Incidentally, these experiments included a number of cell lines—the now famous cell lines—studied by Dr. Gartler, and it may well be that he has made a very fundamental observation of considerable biological interest. Despite their many similarities in addition to their similarities in enzyme patterns as described by Dr. Gartler, there are distinct biological differences among these cell lines, as adjudged by their heterotransplantability to the Syrian hamster cheek pouch, and it would be most unfortunate if Dr. Gartler's observation were to be summarily discarded in the "wastebasket" of cross-contamination with HeLa cells.

On the other hand, there is little question that normal cells in culture occasionally acquire the biological characteristics used for the definition of experimental malignancy. Dr. Sanford has summarized many reports in the literature describing such observations, and there have been other similar reports which ultimately have proved to have been due to cross-contamination in the laboratory. In view of the profound changes that cells undergo in culture—many of which have been discussed in previous sessions during this Decennial Conference—it is remarkable that neoplastic transformation does not occur more often in culture. As Dr. Sanford mentioned, in some instances such transformation appears to have been mediated by virus, and it is interesting that in most instances, *bona fide* neoplastic transformation has occurred in cultures of rodent cells. One wonders if the frequency of this event in cell cultures of rodent origin may not be related to the indigenous virus flora of rodent tissue. In contrast, the rarity of this event in cultures of human cells may be related to the paucity of virus flora in normal human tissue. My virologist colleagues tell me that the isolation of commensal virus from noninvolved human tissue is relatively rare.

Although I personally share the views expressed by Dr. Hayflick that virus contamination very likely is the cause of so-called spontaneous neoplastic transformation—and it is quite evident that we have neither the necessary knowledge nor the tools available as yet to exclude such possibility with certainty in any given instance—we should not be surprised if such neoplastic transformation occurs without the mediation of virus. Alterations in protein synthesis or its regulation which result in the synthesis of new antigens, or in the deletion of specific proteins; the exchange of genetic information between isologous or homologous cells by hybridization or other means; accidents in cell division; the incorporation of functional nucleic acids from heterologous cells; *Mycoplasma*, or perhaps even bacteria—any agency

which disrupts the normal regulatory mechanisms of the cell could result in neoplastic transformation.

In any case, irrespective of the precise nature of the inducing agent, the present evidence indicates that highly specific alterations, or a series of such alterations, at the molecular level are essential for expression of the biological characteristics used to define neoplastic as opposed to other kinds of transformation. Let us hope that by the next Decennial Conference our abysmal ignorance of the precise nature of these alterations and the nature of their specific metabolic consequences to the cell will have been alleviated.

Dr. Gey: We will now call on Dr. Foulds to give his reflections on what we are doing and what he is thinking about.

Dr. Foulds: Cell transformation without identified "cause" recalls the phenomena of "carcinogenesis without a carcinogen" *in vivo* and "induction without an inducer" in embryos. The important question is whether any specific stimulus is required. Dr. Hayflick has referred to the high frequency of transformation of mouse cells. These cells derive from inbred, genetically homozygous animals. The intense inbreeding may be expected to reduce the genetic redundancy and, consequently, increase "noise." Conceivably, transformations may be the consequence of noise and may not depend on a consistently operating virus or other specific stimulus but on disturbances of biological systems brought about by a variety of minor and more or less random upsetting circumstances.

Dr. Hayflick, Dr. Barski, and others have compared cell transformation *in vitro* with progression of neoplasia *in vivo*. Progression refers to a developmental process during which new characters emerge. As far as can be determined, the new characters in progression and transformation are irreversible and heritable or replicable. Transformation shares with progression one notable feature of especial relevance to the present discussion, namely, the *independent progression of characters*. The recognition and analysis of progression depend on the study of identifiable characters that can be *separately* described or measured. Such characters are independently variable and undergo progression independently of one another and not as sets. This holds good at all levels of analysis down to histocompatibility genes and individual enzyme systems. The structure and behavior of neoplasms depend on a multitude of independently variable characters. Probably no two tumors—or transformed cell lines—are exactly alike in every particular. The independent progression of characters results in a *dissociation* of characters that in normal cells are grouped together in sets. These considerations and others indicate the dangers inherent in overgeneralization from special cases of neoplasia.

Some of the new characters that emerge during transformation or progression are irrelevant to the neoplastic process itself, being no more than incidental consequences of it. Others are questionably useful in the recognition of neoplasia or in the distinction between benign and malignant neoplasia. "Malignancy" is essentially an abstract concept based on medical practice; it is not a biological entity. It depends on many independently variable and dissociable characters, none of which provides a reliable criterion of malignant growth. Transplantability is not, and never has been, a reliable criterion; Leo Loeb transplanted an unquestioned *benign* mammary fibroadenoma of a rat in 1916 and many others have done so since. When successful, transplantation demonstrates the replicability of cell characters; the criterion of "malignancy" is the subsequent malignant behavior of the transplanted cells. Transplantation may fail for reasons other than genetic incompatibility or lack of "malignancy," as Dr. Sanford has described.

Professor Abercrombie suggested that lack of "contact inhibition" was correlated with invasiveness, which, as he pointed out, is but one of the independently variable characters contributing to malignant neoplastic growth. Others have been less restrained and Dr. Sanford has drawn timely attention to some disquieting exten-

sions. As she noted, the original definition of contact inhibition has been altered and loss of contact inhibition has become equated with multilayered growth *in vitro*, which in turn has been accepted as a primary criterion of neoplastic transformation, a change from a monolayer to multilayered growth being considered synonymous with transition from a normal to a neoplastic cell population. Dr. Sanford has ably discredited such assumptions. By an insidious process a possibly useful, though limited, "correlation" has become a "criterion" and threatens to become, with disastrous results, a "definition." The basic fallacy lies in *inferring*, without any foundation of observation, the presence of various characters that an observer expects to be associated with one he has been able to recognize. The presumed correlations between, for example, contact inhibition or transplantability and neoplastic behavior, whether benign or malignant, are at best imperfect and at worst dangerously misleading.

As a matter of hard experience, no change attributable to abnormalities of *structural* genes or their products is consistently demonstrable in neoplastic cells. The prevalence of dissociation of characters in neoplasia suggests that dissociation *per se* may be an important or even fundamental criterion. Dissociation is credibly attributable to disturbance of regulatory mechanisms, but its nature is unknown. The irreversibility and replicability of the characters established by progression or transformation suggest a genetic basis implicating, perhaps, the regulator genes. The genetic fault, if there is one, is not necessarily or even probably a mutation, unless that term be stretched far beyond its historical and usual meaning; it might be more akin to the *paramutation* of Brink or the *treption* of Serra and entail a change in the ancillary materials of the chromosomes without alteration of the genetic DNA.

Dr. Hayflick: When Dr. Gey called me to discuss my presentation, he wanted me to be sure to put "ginger" into it. I would say that the right bait was cut and held out.

As Dr. Beard conjectured to me privately, viruses cannot be expected to be entirely responsible for the process we call spontaneous transformation *in vitro*. The point I tried to make is that despite attempts to reproduce this event *in vitro* by other than viruses, that is with chemical carcinogens, or radiation and, despite some positive reports in the literature, I think there is a question in most of our minds about the validity and, in fact, reproducibility of some of these results. This dichotomy really impresses me at the moment.

Dr. Sanford: I am glad Dr. Foulds has emphasized some of the problems in defining this neoplastic transformation.

Dr. Foulds: The hypothesis is beautiful. I started with this idea myself that integration of the virus into the cells is most attractive. The only weakness is there is no evidence for it!

I must confess I view the other point with bias. I have been hearing, at intervals for over 30 years, your plea for patience in this temporary difficulty in discovering human tumor virus *in vivo* and *in vitro* and my reaction has tended to drift from tolerance toward allergy.

Dr. Gey: This has been implicated in neoplasia.

Dr. Foulds: I won't say it never happens, but as a universal order, I agree with you that there may be many other things that come through viruses now in some circumstances, but we can't find what we believe.

Dr. Gey: Dr. Barski, Dr. Foulds has brought up your name. Have you any reflections?

Dr. Barski: I think we have many points of agreement, and, especially, considering the variability in time of tumors *in vivo* and the cell lines *in vitro*. We can imagine quite easily that the evolution of some stem cell lines *in vitro* is starting and continuing *in vitro* in a way which cannot be achieved *in vivo*. I mean that we have an

area of nonmalignant growth which can be evidenced *in vitro*, but not, of course, *in vivo*.

Dr. Fogh: In relation to the possible viral origin, so-called spontaneously transformed cells which has been indicated or emphasized by Dr. Hayflick, I want to make two brief comments. About 8-10 years ago we did some experiments with amnion cells which we really believed at that time gave some indication that an isolate, either from already spontaneously transformed cells or HeLa cells, caused changes and sometimes gave rise to established lines of amnion cells. This was published. And, true enough, we weren't able to reproduce it. But it was somewhat related to the demonstration a few years later by Dr. Edwards and me of virus-like particles in our FL line of spontaneously transformed human cells.

In view of the recent data, which we may or may not believe, on virus particles with Burkitt cells, I suggest it was obvious at that time that these virus-like particles were only visible in old cells in bad shape. Now, we have, at least for the time being, given up more experiments in this direction. Perhaps one day we ought to reevaluate them.

I want to mention the systems we have recently worked with, the data we have presented in Denmark, namely, SV40 transformation of human amnion cells. We have here a system in which we are able to compare definitely virus-transformed cells with SV40 with the various spontaneously derived lines which have occurred over the years. Those lines divide, become passable, and present a number of the characteristics of continuous lines. But there are obvious differences, too. There are differences in morphology which are very clearly visible. They are not the same cells, SV40-transformed and amnion cells and "L" cells. There are differences in ultrastructure and biosynthesis and chemical methods.

The virus-transformed cells in our hands have now died after 18-25 passages, whereas the continuously transformed cells have been carried for up to 11 years. The virus-transformed cells are not tumor-producing to the extent that these spontaneously transformed cells are. In the systems we have examined, which are primarily hamsters, we never have had evidence of tumors. The transformed cells contain the T antigen which, of course, the spontaneously transformed cells do not.

So here is a virus which has caused a transformation of a human cell that apparently is not the one causing the spontaneous transformation. Much more work is needed, but it indicates an excellent system.

Dr. Cailleau: I have been working with AKR mouse leukemia cells for the past 5 years. These leukemia cells seem to defy some of the criteria, as noted by Dr. Hayflick and others, in that the chromosome number has remained 40 and to all appearances "normal." The cells all contain viruses and yet their malignancy or transplantability, or whatever word one wants to use, has diminished in tissue culture. I am afraid this merely shows that no matter what you are working with, someone else is going to be getting just the opposite result.

Dr. Yohn: Regarding the hypothetical model introduced by Dr. Hayflick that the DNA genome of the virus might be integrated directly into the host genome, I don't think we can review the evidence. Dr. Foulds has suggested that we don't have any direct evidence. Earlier in the Conference, we heard evidence for the persistence of at least long-lived messenger RNA. Some neoantigens are induced without the synthesis of viral DNA. Hence we are faced with the problem that perhaps there is a long-lived messenger introduced with the DNA viruses also.

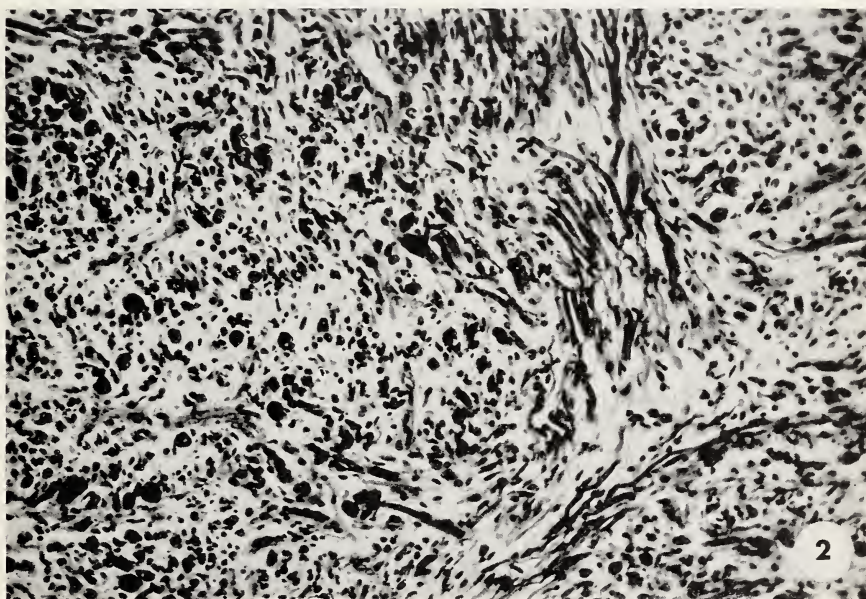


FIGURE 1.—Section of nodule in Syrian hamster cheek pouch 7 days after implantation of 1.0×10^4 cells of cell line derived from human neoplastic tissue (KB-E-CCRF, a subline of KB). Hematoxylin and eosin. $\times 140$

FIGURE 2.—Section of nodule in Syrian hamster cheek pouch 7 days after implantation of 1.0×10^4 cells of cell line derived from human non-neoplastic tissue (Detroit 173B). Hematoxylin and eosin. $\times 140$

"Spontaneous" Neoplastic Transformation of Cells *In Vitro*: Some Facts and Theories¹

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SUMMARY

Chemical carcinogens and viruses have been postulated as causes of "spontaneous" neoplastic transformation of cells *in vitro*. However, cell strains cultured in chemically defined medium and cells that appear from present evidence to be free of detectable virus have also undergone neoplastic conversion. In one culture system, cells from mouse embryo or kidney tissue of newborns transformed reproducibly after a predictable period of culture. Rat embryo cells appeared to be more stable with respect to neoplastic transformation than mouse cells; Syrian hamster embryo cells were intermediate in stability. Cells from adult mouse tissues underwent neoplastic conversion later than those from tissues of embryos and newborns. For the first time, a component of the culture medium, fetal calf serum, was found to influence the time of neoplastic conversion. This component also resulted in cell populations showing less structural alteration in

chromosomes. Some of the evidence presented appears contradictory to current hypotheses and concepts. One concept is that all continuous lines of cells are neoplastic and that only cells that are neoplastic can grow as continuous cell lines. Results from our method of testing tend to disprove also the hypothesis that all aneuploid or heteroploid continuous cell lines have acquired the properties of cancer cells and can be considered equivalent to transplantable tumors. The concept that the morphologic alteration described as "piling up" of cells or change from monolayer type growth to multilayered growth is caused by loss of "contact inhibition" appears to have inadequate experimental support. Further, we have found that cells showing monolayer growth are not always non-neoplastic and cells growing in multilayers are not always neoplastic as tested by *in vivo* implantation of cells.—Nat Cancer Inst Monogr 26: 387–418, 1967.

THE SUBJECT of this Conference is the relationship between the cultured cell or tissue and its *in vivo* progenitor. The objective is to evaluate tissue culture as a means for studying the structure and responses of cells

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² Tissue Culture Section, Laboratory of Biology.

³ National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

in the living organism. One response of cells that may occur *in vivo* or *in vitro* is neoplastic transformation. In both higher plants and animals, cells from a variety of tissues, under certain inductive stimuli, enter a phase of continuous uncontrolled growth and division, forming tumorous masses detrimental or fatal to the organism. As *in vivo*, so *in vitro* tissue cells explanted to culture from both plants and animals may undergo such transformation. The transformed cells, when returned to the type organism from which they were derived, tend to grow progressively, frequently causing death of the host. The tumors arising from the transformed animal cells usually behave like malignant neoplasms and are frequently indistinguishable from many invasive anaplastic tumors of *in vivo* origin. Neoplastic transformation of cells *in vitro* permits study of the cell changes under controlled experimental conditions.

Although neoplastic transformation of mammalian tissue cells *in vitro* can be induced by certain carcinogenic agents such as tumor viruses [for review, see (1)], there are numerous reports of neoplastic transformation *in vitro* where no known carcinogenic chemical or virus has been deliberately added to the culture. These neoplastic transformations of unknown cause are referred to as "spontaneous." The frequency of such occurrences is exceedingly high in long-term cell strains—so high that some investigators have implied that all continuous lines of cells are neoplastic—and conversely, only cells that are neoplastic can grow as continuous cell lines. In fact, the alteration of a cell strain to a continuous line has been considered oncogenesis *in vitro* (2). Some data bearing on this hypothesis will be considered later.

The phenomenon of "spontaneous" neoplastic transformation of mammalian tissue cells was first reported in 1941 by Gey (3, 4) in cultures of rat fibroblasts and soon after by Earle (5) in cultures of mouse fibroblasts. In the past 25 years, reports of "spontaneous" neoplastic transformation have appeared from many laboratories in cell strains derived from a wide variety of tissues from different strains of several different species of animals. Some of these are listed in table 1. The type of serum or plasma used in the culture medium is included for reasons to be considered later. Most of the tumors developing *in vivo* from the tissue culture implants have been diagnosed as sarcomas. A notable exception is the cell strain derived from C3H mouse liver which gave rise to tumors diagnosed as anaplastic carcinomas resembling hepatoma (9). Neoplastic conversion appears to be a reproducible response of C3H mouse fibroblasts to certain culture conditions; almost every strain of cells established *in vitro* has given rise to at least some sarcomas when assayed *in vivo*.

Both chemical carcinogens and viruses have been postulated as causes of "spontaneous" neoplastic conversion. Much of the work of our laboratory has been directed toward a better control of the chemical environment of the cultured cell in order to eliminate the accidental introduction of

any carcinogenic chemical. Rubber closures for culture flasks were considered possible sources of contamination, since additives used in fabrication of some forms of commercial rubber could possibly be carcinogenic. Replacement of rubber stoppers with those made from silicones having a purified silicon dioxide filler did not prevent the neoplastic transformation in cultured cell strains. The development of chemically defined culture media in our laboratory has made possible the continued rapid growth of many strains of cells from mouse, hamster, monkey, and human tissues without addition of any undefined supplement such as serum, proteins, peptones, or hydrolysates (35). Three strains of C3H mouse embryo cells were initiated and maintained by Andresen, Evans, and associates in chemically defined medium without addition of any hormones or enzymes such as trypsin (15, 16). All three strains have become neoplastic, giving rise to sarcomas on implantation *in vivo*. One of these can be considered an established strain or continuous line routinely transplantable at 7-day intervals. The other two have not been successfully subcultured to date. Waymouth (20, 21) initiated three strains of cells from C57BL mouse fetal lung tissue in chemically defined medium supplemented with a small amount of insulin. Two strains were treated initially with enzymes; the third was initiated and subcultured by mechanical means only. All three strains after 17 months to 3 years *in vitro* were found to be neoplastic, producing slow-growing sarcomas, rapid-growing sarcomas, or differentiated osteogenic sarcomas when implanted *in vivo*. Thus, it appears that even when cells are cultured in a chemically controlled environment without addition of any hormone or enzyme as in the first study, neoplastic conversion still takes place.

The widespread occurrence of tumor viruses, especially in mice, suggested that viruses either in the original tissue explant or in serum-containing medium might be the cause of "spontaneous" neoplastic transformation. To explore this possibility further, investigators in our laboratory (36) collaborated with Dr. J. W. Hartley in the examination of our murine cell strains for complement-fixing mouse leukemia viruses. Forty-six cell lines derived from 20 different cell pools were examined either before or after neoplastic transformation. Of the 20 cell pools, 7 gave rise to lines showing presence of leukemia virus. Two of these cell pools had been cloned; the clones are interesting because of their widespread use and also because about 10 years ago, Dr. A. J. Dalton (37) observed electron microscopic type C particles in one and, more recently, in both clones. Repeated efforts to establish biologic activity for these particles have been unsuccessful to date. One of these was clone 929 from strain L, initiated in 1940 from subcutaneous connective tissue of an adult strain C3H mouse (38). The other was a clone also derived from subcutaneous connective tissue taken from an adult C3H mouse 10 years later in 1950 (7). The latter clone was subdivided into 8 cell lines in an experiment to determine whether neoplastic transformation would occur at

TABLE 1.—Reports of "spontaneous" neoplastic transformation of cells *in vitro*

Tissue source	Number of cell strains*	Serum or plasma used	References
MOUSE			
C3H or C3Hf			
Subcutaneous.....	2	Horse and chicken.	Earle and Nettleship (5), Sanford <i>et al.</i> (6)
connective tissue	3	Horse.....	Sanford <i>et al.</i> (6-8)
Liver (2-day).....	1	Horse and chicken.	Evans <i>et al.</i> (9)
Skeletal muscle.....	2	Horse and chicken.	Sanford <i>et al.</i> (6)
Skin.....	2	Horse.....	Weaver [see (7)], Rothfels <i>et al.</i> (10)
Parotid.....	2	Horse.....	Sanford <i>et al.</i> (8)
Lung.....	1	Horse.....	Rothfels <i>et al.</i> (10)
Kidney (3-day).....	3	Horse.....	Andresen <i>et al.</i> (11)
(adult).....	3	Horse.....	Rothfels <i>et al.</i> (10)
Embryonic tissues....	9	Horse.....	Evans <i>et al.</i> (12), Sanford <i>et al.</i> (13)
	2	Fetal calf.....	Evans <i>et al.</i> (14)
	3	None.....	Andresen <i>et al.</i> (15, 16)
C57BL			
Lung.....	1	Horse, calf.... fetal calf.	Barski and Cassingena (17)
Embryonic tissues....	1	Horse.....	Sanford (18)
	1	Calf.....	Habel (19)
Fetal lung.....	3	None.....	Waymouth (20, 21)
I			
Subcutaneous.....	1	Horse.....	Sanford (18)
connective tissue			
ALB-M2T			
Embryonic tissues....	1	Horse.....	Sanford <i>et al.</i> (22)
A			
Cardiac muscle.....	1	Fetal calf.....	Dipaolo (23)
Strain not stated			
Embryonic skin.....	1	Horse.....	Levan and Bieseke (24)
RAT			
Philadelphia albino			
Subcutaneous.....	1	Human cord, chicken.	Gey (3)
connective tissue			
Wistar			
Subcutaneous.....	1	Rabbit.....	Benevolenskaya and Staroverova (25)
connective tissue	1	Horse.....	Staroverova (26)
Albany			
Embryonic tissues....	1	Horse.....	Sanford <i>et al.</i> (22)
Strain not stated			
Embryonic tissues....	4	Horse, calf, human cord	Sandritter <i>et al.</i> (27)
HAMSTER			
Syrian			
Kidney (1-day).....	1	Calf.....	Stoker and MacPherson (28)
Embryonic tissues....	5	Horse.....	Sanford <i>et al.</i> (13)
	1	Calf.....	Sanford <i>et al.</i> (13)
	1	Calf and fetal calf	Gotlieb-Stematsky <i>et al.</i> (29).
Chinese			
Embryonic tissues....	1	Not stated....	Yerganian <i>et al.</i> (30)

See footnote at end of table.

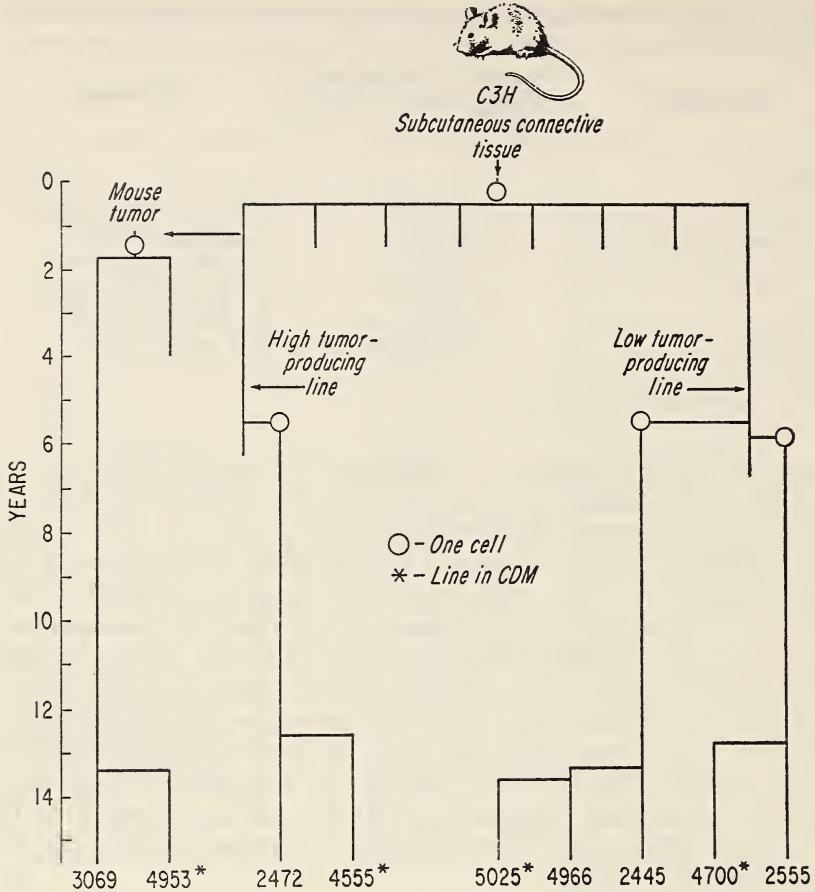
TABLE 1.—Reports of "spontaneous" neoplastic transformation of cells *in vitro*—Con.

Tissue source	Number of cell strains*	Serum or plasma used	References
HUMAN			
Liver.....	1	Human.....	Moore <i>et al.</i> (31), Southam <i>et al.</i> (32)
Kidney.....	1	Human.....	Moore <i>et al.</i> (31), Southam <i>et al.</i> (32)
Conjunctiva.....	1	Human.....	Moore <i>et al.</i> (31), Southam <i>et al.</i> (32)
Skin.....	1	Human.....	Van Scott <i>et al.</i> (33)
Amnion.....	1	Human.....	Fogh and Hok (34)

*Each cell strain is from a different animal.

random or simultaneously in independent lines derived from one cell (text-fig. 1). The transformation appeared to be random. Two lines were continued in culture: a high tumor-producing line that grew rapidly into malignant tumors in almost all animals given injections and a line that produced no tumors and later a low incidence, referred to as a low tumor-producing line. Differences between these two lines and their derivatives have persisted through the years of culture. A tumor arising from the high-tumor line was explanted to culture and cloned. From this clone and the original lines, 9 clones or derivative lines have been continued on serum-containing medium or on chemically defined medium NCTC 135 (35). All 9 tested positive for complement-fixing mouse leukemia virus and all examined by Dr. Dalton, and more recently by Dr. W. T. Hall (39), contain numerous type-C particles, more in the low-tumor line than the high.

We have tentatively concluded that mouse leukemia viruses present in the original mouse tissues explanted in 1940 to initiate strain L and in 1950 to initiate the high- and low-tumor lines have persisted through the single cell isolates for the past 26 and 16 years *in vitro*, respectively. Although it is uncertain whether these viruses played any role in the neoplastic conversion, the virus infection possibly accounted for the immunologic alteration we have observed in the two clones (40, 41). Both high- and low-tumor lines and clone 929-L were found to be antigenic to mice of the inbred strain C3H from which the cells originated. In these mice, the high- and low-tumor lines cross-immunized against each other and also against clone 929-L cells. The derivative of clone 929-L, strain 2071 (42), that has been carried in chemically defined medium for the past 11 years gives a negative test for complement-fixing mouse leukemia viruses; also, from Dalton's observations (37) it appears to be free of the virus particles.



TEXT-FIGURE 1.—Lineage of high- and low-tumor-producing lines and clones. Subcutaneous connective tissue of an adult C3H mouse was explanted to culture November 30, 1950, and 97 days later one cell was isolated. During the first 5½ years, cells were cultured in a mixture of 20% chick embryo extract, 40% Earle's saline, and 40% horse serum. Chicken plasma clot or perforated cellophane formed the substrate. Subsequently, cells were cultured in medium NCTC 109 or 135 (35) plus 10% horse serum or in unsupplemented medium 135 (CDM).

A third strain of cells that gave a positive test for leukemia virus was one of the three C3H mouse embryo cell strains initiated in chemically defined medium referred to earlier that has become established as a continuous cell line. Again, it is uncertain whether the virus played any role in the neoplastic conversion.

Twenty-two cell strains from 13 of the 20 cell pools gave negative tests for complement-fixing mouse leukemia viruses. Eight of these were also tested by Dr. J. C. Parker (Microbiological Associates, Inc., Bethesda,

Md.) for a group of 10 murine viruses (43). All tests were negative. Sixteen of these 22 cell strains were either neoplastic or have undergone neoplastic conversion since test. It thus appears from evidence presently available that "spontaneous" neoplastic transformation can occur in the absence of detectable leukemia or certain other murine viruses.

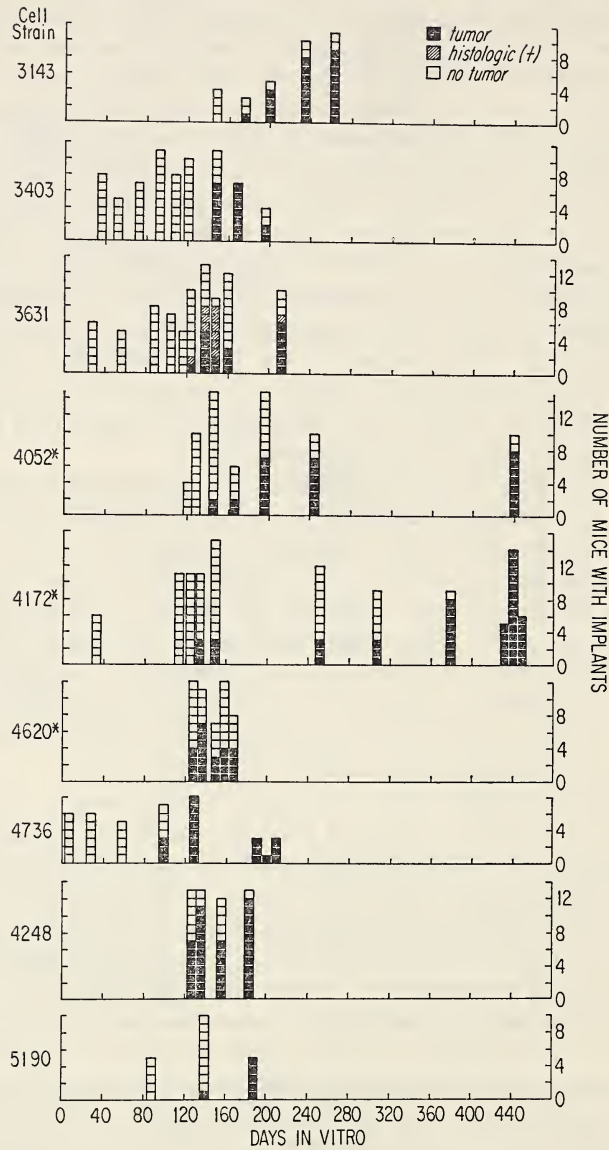
Although it was early recognized that "spontaneous" neoplastic transformation is a reproducible response of C3H mouse fibroblasts to certain culture conditions (6), the time of conversion appeared to be unpredictable. More recently, Evans, Andresen, and associates (11, 12) found that in 9 strains of C3H mouse cells derived from minced embryos or kidneys of 3-day-old animals, grown in NCTC 135 with 10% horse serum and assayed by implantation in the anterior chamber of the mouse eye, malignant transformation became manifest after approximately the same period of culture *in vitro* (text-fig. 2). This period ranged from 98–188 days. The reproducibility of this response with respect to time in culture is even more remarkable when one considers the heterogeneity of cell types in minced whole embryo or kidney from 3-day-old mice.

This early and reproducible response may be characteristic of cells from the embryo or newborn mouse only. Embryo cells from the Albany rat appeared to be more stable with respect to neoplastic conversion (text-fig. 3). We initiated several strains of rat embryo cells *in vitro* because the chromosomes of rat cells were reputedly less subject to alteration *in vitro* than those of the mouse. Intraocular implants to date have given rise to only two tumors, one of which was not successfully transplanted; the other (from strain 4965) is a very slow-growing sarcoma (22). No tumors have as yet arisen from the few implants of strain 5066 cells which were initiated and maintained in chemically defined medium.

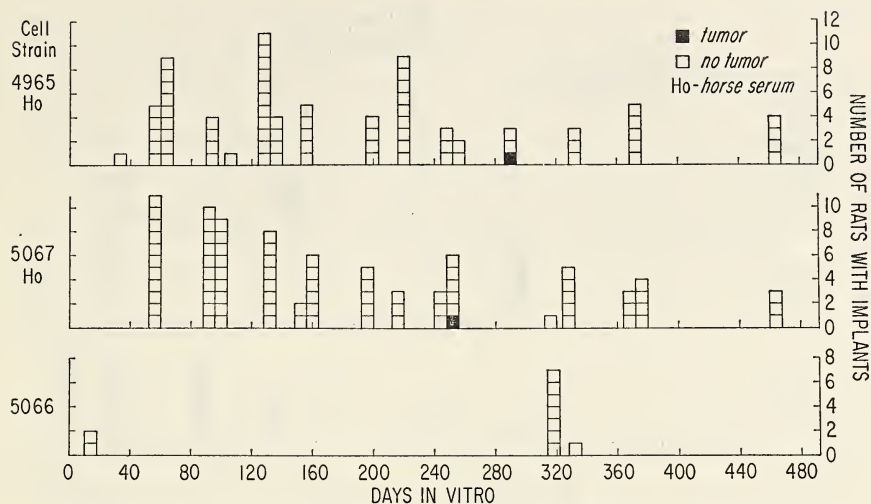
Syrian hamster embryo cells appear to be intermediate in stability with respect to neoplastic conversion (text-fig. 4). The neoplastic response was reproducible, but later and more variable in time of occurrence (13). In general, fibroblast-like cell strains (such as strain 4109) underwent neoplastic conversion sooner than epithelial-like cell strains (such as strain 4486). It may be noted that some of the earlier assays were by inoculation subcutaneously into newborns or intramuscularly into adult animals. The latter were usually X-irradiated to lower their immune defenses and thus increase the sensitivity of assay, since the Syrian hamster is not inbred. All tumors were sarcomas transplantable to unconditioned adults. With two exceptions, these cell strains were initiated and maintained in NCTC 135 and 10% horse serum. Strain 5202 was grown in Eagle's minimum essential medium with "nonessential" amino acids⁴ supplemented with 10% calf serum.⁵ Strain 5204, which is still non-neoplastic, was grown in NCTC 135 and 10% fetal calf serum. The effect of fetal calf serum in preventing or delaying neoplastic conversion will be discussed later.

⁴ Powdered form obtained from General Biochemicals, Chagrin Falls, Ohio.

⁵ Obtained from Flow Laboratories, Rockville, Md.



TEXT-FIGURE 2.—Results of intraocular implants of cell strains derived from C3H mouse embryos or kidneys* of 3-day-old animals. Cells were grown in NCTC 135 and 10% horse serum. The number of mice with implants is plotted against days *in vitro*. Tumors were diagnosed as sarcomas and were transplantable to unconditioned adult C3H mice.

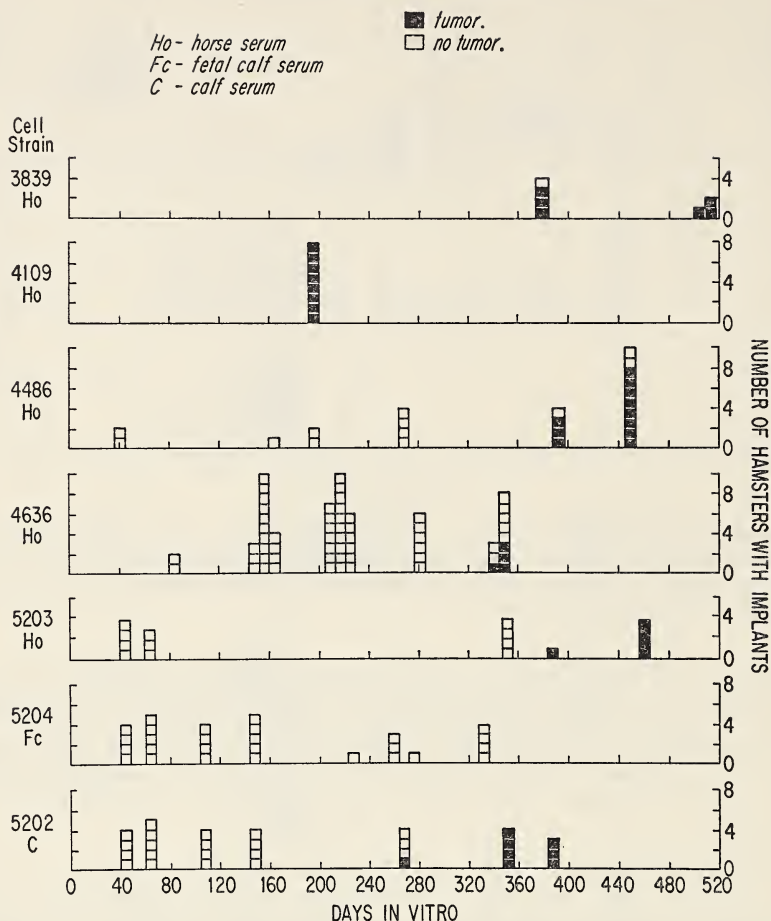


TEXT-FIGURE 3.—Results of intraocular implants of strains of Albany rat embryo cells cultured in NCTC 135 and 10% horse serum or in unsupplemented NCTC 135 (strain 5066). All animals receiving intraocular implants and failing to develop tumors were observed for a year.

Cells derived from adult mouse tissues may undergo neoplastic conversion later than those from embryonic or newborn tissues, as is suggested by these results of intramuscular implants into X-irradiated mice (text-fig. 5). Cell strains from adult tissues of C3H and I mice are compared with cell strains from embryonic tissues of C3H and C57BL mice (8, 12, 18).

Recently, Evans and Andresen (14) observed that a strain of C3H mouse embryo cells initiated in NCTC 135 and 10% fetal calf serum instead of horse serum failed to undergo neoplastic conversion even after 777 days *in vitro* (text-fig. 6). This cell strain (4823), currently in the 124th transplant generation, can be considered a continuous cell line. Cultures are split 1–4 every 7 days. This non-neoplastic cell strain, as well as others reported in the literature (4, 6, 9, 17, 25), bring into question the hypothesis referred to earlier that all continuous cell lines are neoplastic and that only neoplastic cells can grow continuously *in vitro*.

A second strain of C3H mouse embryo cells (5094) grown in medium 135 supplemented with fetal calf serum became neoplastic only after 418 days *in vitro*, whereas cells of the same pool grown in medium 135 and horse serum were neoplastic by 172 days. We have confirmed this effect of the fetal calf serum with a third cell strain, 5143, which was still non-neoplastic after 412 days, whereas cells of the same pool cultured in horse serum medium, strain 5144, became neoplastic between 139 and 181 days *in vitro*. One exception in the study by Evans and Andresen (14) is a



TEXT-FIGURE 4.—Results of implanting *in vivo* cell strains derived from Syrian hamster embryos. Cells were grown in NCTC 135 and 10% horse serum, except as indicated in text. The first 4 hamsters receiving cells of strain 3839 and 3 of the 5 receiving cells of strain 4109 were newborns less than 48 hours old, and were given subcutaneous injections. The remaining animals of these two series were X-irradiated adults that received injections intramuscularly. Cells of strains 4486 and 4636 were injected intramuscularly into X-irradiated adults or, where more than one animal was used, into an equal number of X-irradiated and unirradiated adults, except for the last injection set. The last injections of these cells and of cells of strains 5202, 5203, and 5204 were intraocular.

strain of cells (4933) which became neoplastic at 56 days *in vitro*. This is the only strain in this group that gave a positive test for mouse leukemia virus. Possibly, the viral infection may account for the early neoplastic conversion.

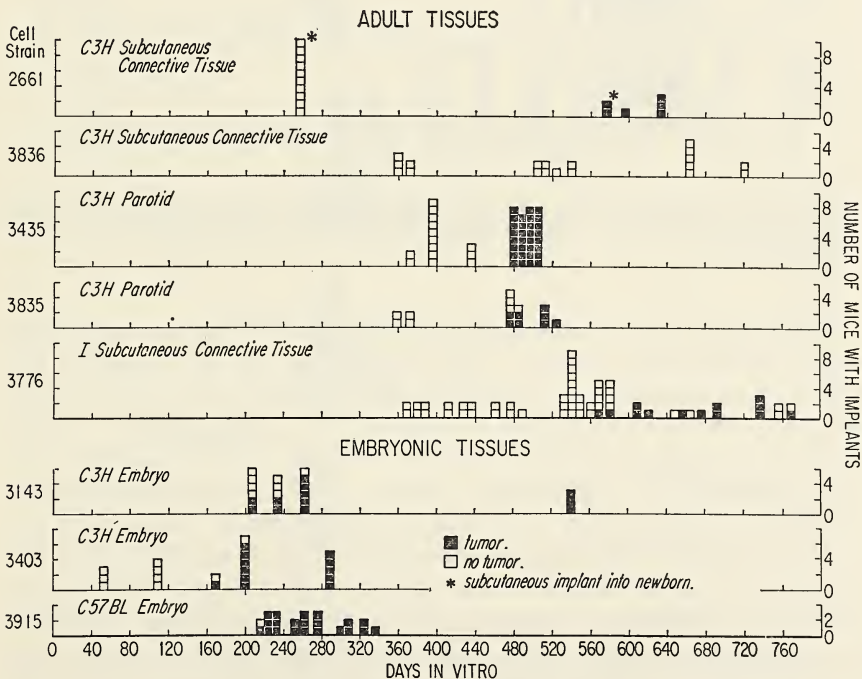
For the first time, a component of the nutrient medium has been shown to influence the time of neoplastic conversion. Further studies are in prog-

ress to identify the active material and the mechanism by which neoplastic conversion is delayed or prevented.

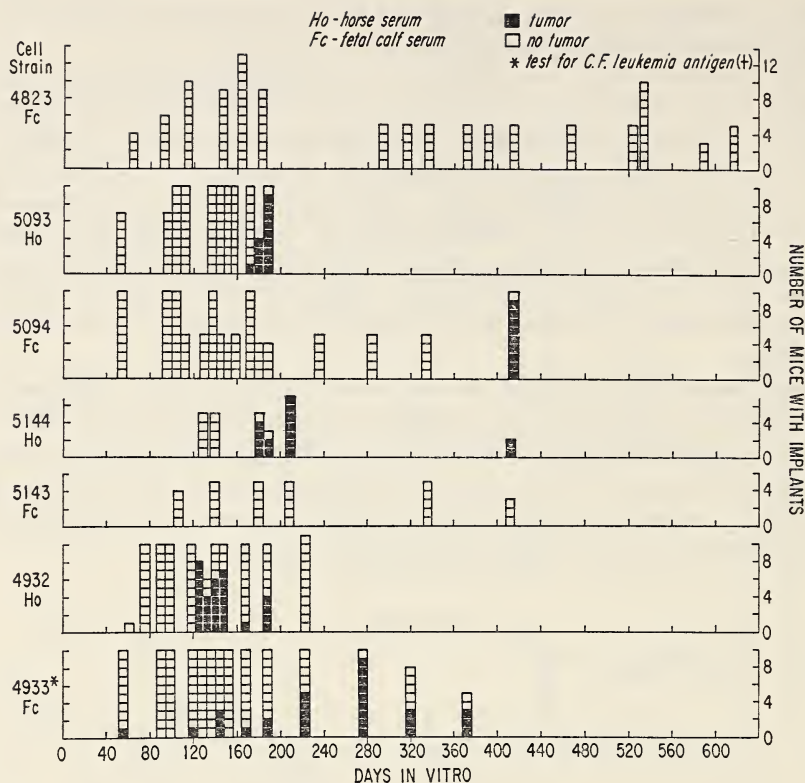
The fetal calf serum was apparently not interfering with transplantability of the cells. Converted cells in horse serum medium transferred to fetal calf serum medium grew as rapidly into tumors when implanted in mice as the cells maintained continuously in horse serum medium (14).

Cultures transferred from fetal calf serum medium to horse serum medium required the same period of growth in horse serum before neoplastic conversion as cultures initiated and grown continuously in horse serum (14). This observation suggests that some factor in horse serum absent from fetal calf serum is carcinogenic when exposure is of sufficient duration.

An alternative hypothesis is that some factor in fetal calf serum stabilizes the cells preventing neoplastic change. The occurrence of neoplastic change in cells grown on chemically defined medium without serum supports this concept. However, as noted earlier, one of the cell strains on chemically defined medium is infected with a leukemia virus; the other strains have not yet been tested for viruses.



TEXT-FIGURE 5.—Results of intramuscular implants of cell strains derived from mouse adult and embryonic tissues. Cell strains 3143, 3403, and 3631, referred to in text-figure 2, were assayed by intramuscular as well as intraocular routes (12). All tumors were sarcomas transplantable to unconditioned adults.



TEXT-FIGURE 6.—Results of intraocular implants of cell strains derived from C3H or C3Hf embryos and grown in NCTC 135 and horse serum (Ho) or fetal calf serum (Fc).

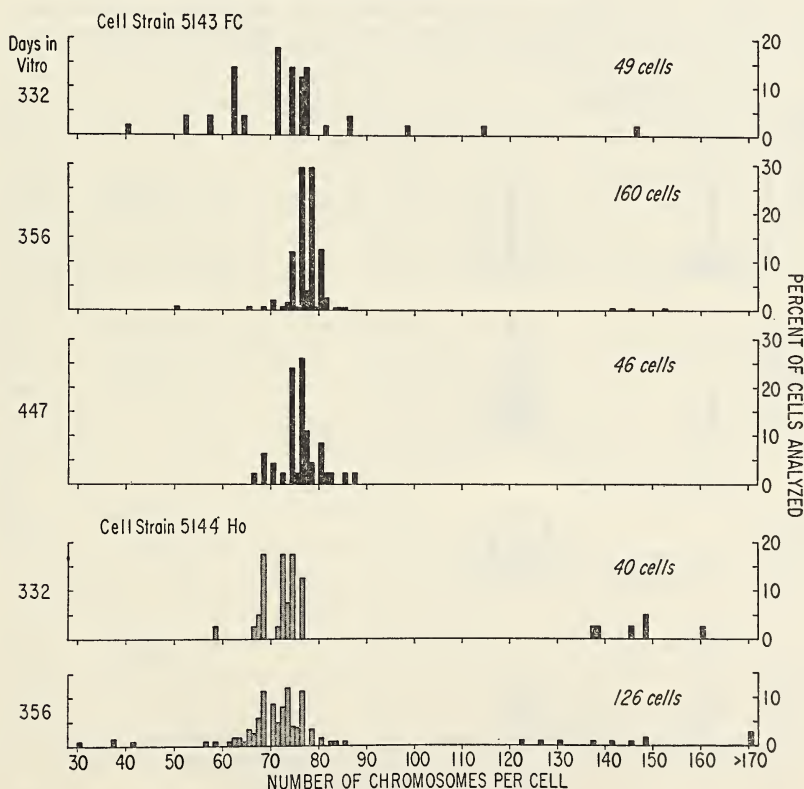
Chromosomes of the non-neoplastic cell strain 5143, grown on fetal calf serum-supplemented medium, were studied by Dr. R. Parshad (44). Chromosome numbers in these cells were compared with those of the neoplastic strain 5144, cultured from the same pool of cells on horse serum-supplemented medium (text-fig. 7). Both cell strains were aneuploid, exhibiting a wide range of chromosome numbers predominantly above the diploid number of 40. These observations, as well as others in the literature (17, 25), force us to modify the hypothesis that cell strains that have become aneuploid or heteroploid continuous cell lines have acquired the properties of cancer cells and can be considered equivalent to transplantable tumors (2). In fact, cell strain 4823, which still tests as non-neoplastic after more than 2 years *in vitro*, has been heteroploid since the first 200 days *in vitro* (16).

It is possibly significant that cells in the horse serum-supplemented medium as compared with the cells in fetal calf serum medium exhibited

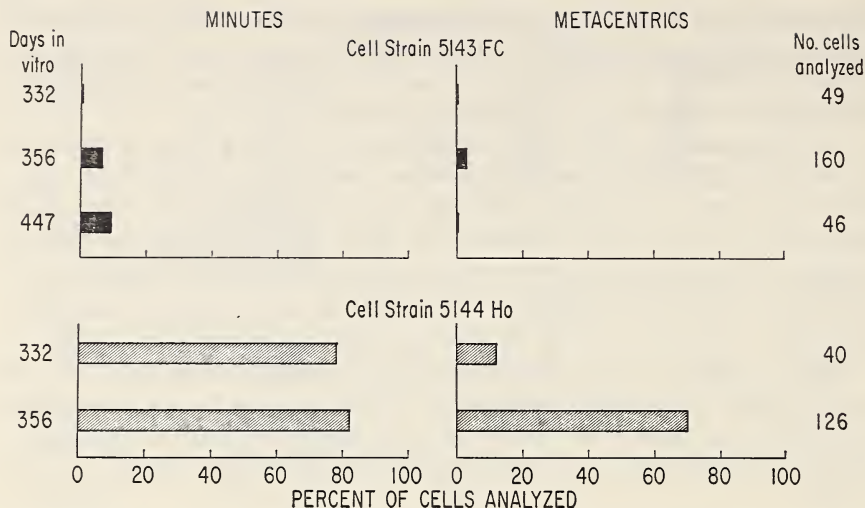
more structural alterations in chromosomes. A large number of cells contained minute and biarmed or metacentric chromosomes (text-fig. 8). These alterations in chromosome size and shape indicate considerable breakage, rearrangement, and possible deletions.

Chromosomes of another pair of cell strains derived from embryos of a germfree ALB M2T mouse were also studied by Pr. Parshad (44). Cells had been cultured for a shorter period in medium 135 with fetal calf serum or horse serum (text-fig. 9). Both cell strains showed a wide range of chromosome numbers. After this shorter period of culture more structural alterations appeared in the cells cultured on horse serum-supplemented medium than in those grown in fetal calf serum (text-fig. 10).

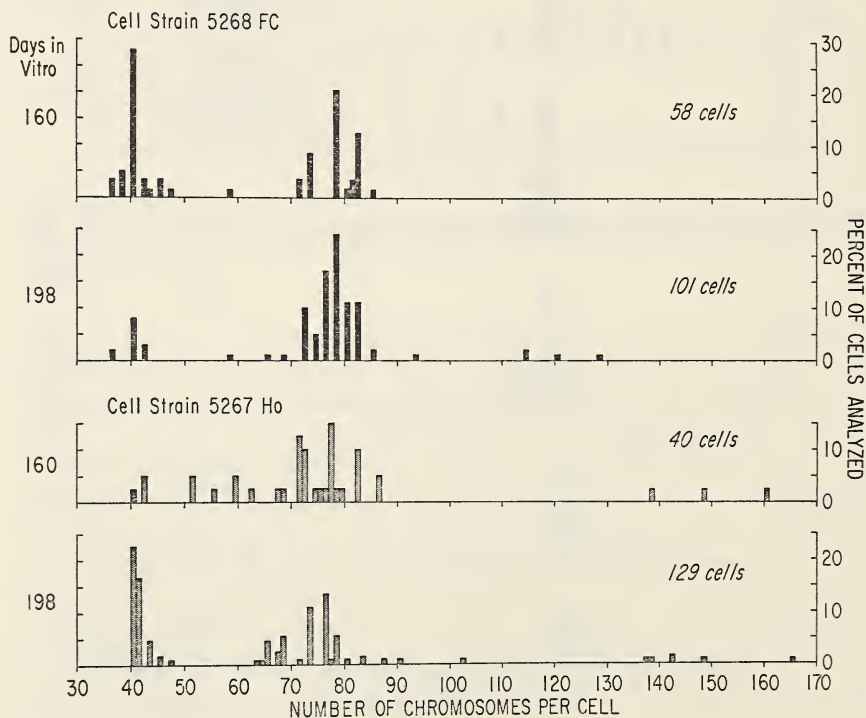
Similar effects of horse serum and fetal calf serum were noted in cell strains initiated by Evans, Andresen, *et al.* and studied by Mr. J. Jackson (45). However, as time in culture was extended, the number of cells showing structural alterations in chromosomes also increased. Cell lines grown



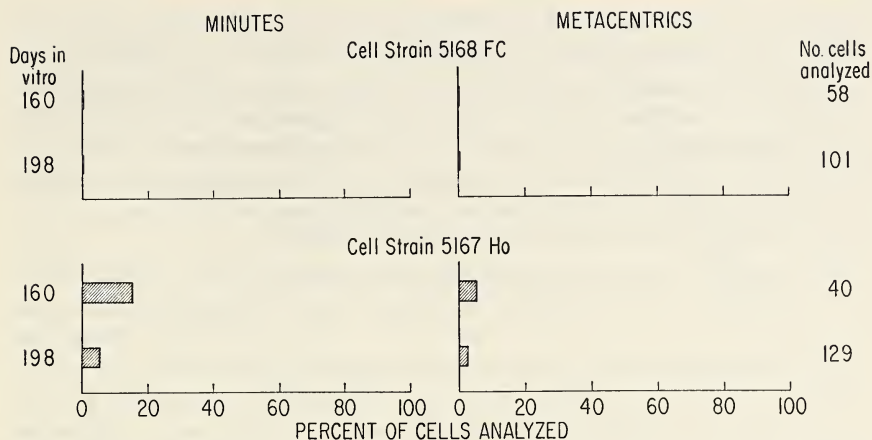
TEXT-FIGURE 7.—Frequency distribution of chromosome numbers in non-neoplastic cell strain 5143 grown on NCTC 135 and fetal calf serum (FC) and neoplastic cell strain 5144 grown on medium 135 and horse serum (Ho).



TEXT-FIGURE 8.—Percentage of cells with minute or metacentric chromosomes in cell strains grown on NCTC 135 and fetal calf serum (FC) or horse serum (Ho).



TEXT-FIGURE 9.—Frequency distribution of chromosome numbers in cell strains 5268 and 5267 grown on NCTC 135 and fetal calf serum (FC) and horse serum (Ho), respectively.



TEXT-FIGURE 10.—Percentage of cells with minute or metacentric chromosomes in cell strains grown on NCTC 135 and fetal calf serum (FC) or horse serum (Ho).

in fetal calf serum-supplemented medium eventually showed as much alteration as cells in horse serum medium. Rothfels and associates (10) also reported that strains of C3H mouse embryo cells grown in medium supplemented with fetal calf serum maintained a high degree of euploidy longer than strains in the same type medium supplemented with horse serum. Hsu and associates (46) described a strain of cells from C3H mouse bone marrow that maintained for 9 months a karyotype grossly indistinguishable from that of the normal mouse when cultured in fetal calf serum.

The mechanism of this serum effect on structural alterations in chromosomes is under study. What relationship, if any, the structural alterations in chromosomes have to neoplastic transformation is not clear. The results of our studies and those of others (17, 24, 26, 30) are sufficiently suggestive to warrant additional exhaustive analysis for possible correlations with neoplastic transformation.

Some investigators have interpreted certain responses of cells *in vitro* to tumor viruses and chemical carcinogens as "indicators" of neoplastic transformation [for review, *see* (1)]. One of these responses is a specific morphologic alteration referred to as lack of orientation of cells with respect to each other, "criss-cross" growth, piling up of cells to form multilayered interwoven networks, and change to fusiform-cell shape. In contrast, the cells in control cultures tended to grow as flat monolayers with more nearly parallel orientation of cells. These investigators have attributed this morphologic change to a "loss of contact inhibition."

The term "contact inhibition" was applied by Abercrombie and associates (47-49) to the behavior of freshly isolated chick embryo heart fibroblasts. "Contact inhibition" was defined as the stopping of locomotion

tion of a cell in a particular direction by contact with another cell. The visible expression of contact inhibition was cessation of activity in the leading membrane of the cell on contact with another cell and the subsequent adhesion of the two cells. Cells exhibiting contact inhibition formed a monolayer with little or no overlap. Since this phenomenon was observed in freshly isolated chick and mouse fibroblasts but not in mouse sarcoma cells, Abercrombie and associates proposed the hypothesis that malignancy involves the loss or diminution of "contact inhibition."

Subsequently, many investigators have assumed that if cells form a monolayer, the cells are "contact-inhibited" even though they have made no microcinematographic observations for the phenomenon of contact inhibition, *i.e.*, the membrane paralysis, stopping of locomotion, and cell adhesion during the contact of one cell with another. Monolayer growth may result from other conditions. For example, migratory activity of cells associated with a low mitotic rate results in a spreading of the cell population into a monolayer. Certain cell types, particularly cells of epithelial origin, grow in flattened membranous sheets. In certain culture media, such as chemically defined medium, cells adhere so tightly to the glass substrate as to form monolayers even in dense culture. Adding serum to the medium increases migration rates and results in multilayering; the effect is reversible, and under both culture conditions the cells test as neoplastic (50).

It has also been assumed that if cells form a multilayered growth the cells have lost "contact inhibition." In most cases, the existence of contact inhibition as originally defined by Abercrombie and associates has not first been critically established. Further, several other phenomena can result in multilayering, such as decreased adhesion of cells to glass associated with increased rates of mitosis and migration. Many culture conditions and proliferative responses of cells can result in multilayered cultures [for review, *see* (1), *also* (51)].

The term "contact inhibition" as originally defined has thus acquired a new meaning. Cells are contact-inhibited if they grow as a monolayer. Cells have lost "contact inhibition" if they form a multilayered growth. Yet there is still the implication of a cell-to-cell inhibition on contact. Even the inhibition of growth that occurs during the stationary phase of the growth cycle has been attributed to "contact inhibition," but no evidence for a cell-to-cell reaction has been presented (52). A wide array of physicochemical factors are known to operate during this phase to inhibit growth. "Loss of contact inhibition" in its new meaning has gradually come to be regarded as a primary criterion of neoplastic transformation. The change from a monolayer to multilayered growth pattern is considered synonymous with the transition from a normal to a neoplastic cell population.

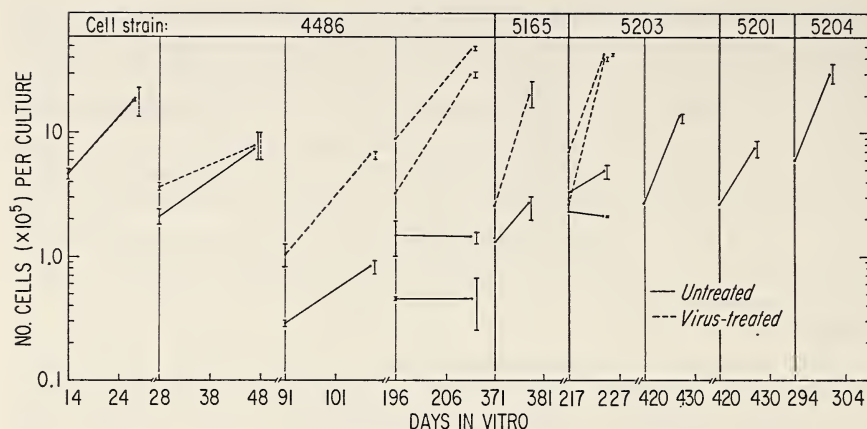
We have examined this hypothesis in the same type of virus-cell culture system used by many of the investigators who have proposed these con-

cepts. This system included the infection of Syrian hamster embryo cells with polyoma virus. We first examined by microcinematography the behavior of normal hamster embryo cells when freshly explanted in medium 135 and 10% horse serum. We observed little or no "contact inhibition" as originally defined by Abercrombie and associates under our culture conditions. Locomotion of cells on contact was not inhibited; cells crawled over and under one another, and we saw no paralysis in the leading membrane of the cell on contact with another cell. Multilayering and "criss-cross" growth patterns also developed, but no rigorous correlation could be made with neoplastic conversion as tested by *in vivo* implantation of cells (13).

After a phase of proliferative activity, hamster embryo cells under certain culture conditions enter a period of reduced growth and proliferation. The virus-infected cells may fail to exhibit this retardation in growth as is shown in the experiments summarized in text-figure 11. Note, particularly in strain 4486, the reduced growth rate with period *in vitro* and the continued high growth rate in the virus-treated cultures. The virus-treated cultures also showed the morphologic response, *i.e.*, piling up of cells, "criss-cross pattern, multilayered growth" generally attributed to "loss of contact inhibition." We concluded from these experiments that the morphologic response to the virus was not the result of loss of "contact inhibition," which was not demonstrable in the first place, but was a proliferative response to virus infection. This proliferative response was associated with decreased adhesion of cells to the glass substrate. The cells showing this response were tested and found to be neoplastic (13). However, such morphologic responses may also be induced in cells already neoplastic [for review, *see* (1)]. In conclusion, the increased rate of cell proliferation induced in a slow-growing cell population by the tumor virus appeared to be the cause of the morphologic response rather than loss of "contact inhibition."

All strains of untreated hamster embryo cells except strain 5204 have ultimately undergone neoplastic conversion. Certain of these cell strains maintained an epithelial-like morphology and grew as flat monolayer cultures. These observations, as well as many others [for review, *see* (1)], force us to modify the hypothesis that "contact-inhibited" cells in the sense of cells showing monolayer growth can be considered non-neoplastic. Also in our experience cells that grow in multilayers may not be neoplastic (13).

After a more extended period of culture *in vitro*, the untreated control cells, for example, strain 5203 (text-fig. 11), gradually assumed a high rate of proliferation and growth. The non-neoplastic strain 5204 tended to grow at the same or slightly higher proliferative rate than the two neoplastic strains 5203 and 5201, which arose from the same pool of embryo cells but were grown in different types of culture medium as noted earlier (text-fig. 4). These observations indicate that non-neoplastic cells may



TEXT-FIGURE 11.—Growth responses of Syrian hamster embryo cells after various periods *in vitro* with and without polyoma virus infection. Each determination is the average of 3–5 replicate cultures except in the last assay on strain 4486 when 2 cultures were used. Cell strains 4486, 5165, and 5203 were cultured in NCTC 135 and 10% horse serum. Polyoma virus was added at 17, 24, and 14 days *in vitro*, respectively. Cell strains 5202 and 5204 were grown in different media as described earlier.

grow almost as rapidly *in vitro* as neoplastic cells. This generalization also held when the cells were grown in the same type of culture medium (13).

Plant tumor cells may also grow *in vitro* at the same rate as their normal counterparts if an enriched culture medium is used. However, in a simple defined medium containing inorganic salts and sucrose, crown-gall tumor cells could grow, whereas normal tissue cells could not. Normal cells could proliferate only when this medium was supplemented with an auxin, cytokinin, mesoinositol, glutamine, asparagine, and cytidylic and guanylic acids.

This difference in nutritional requirements provided a clue for exploring the nature of neoplastic transformation in plant cells. Such clues have not yet been found for animal cells. No distinctive differences in nutritional requirements of normal and neoplastic animal tissue cells *in vitro* have been reported. However, until freshly isolated tissue cells can be readily grown in quantity in chemically defined medium freed from the biologically active components of serum or serum proteins, such nutritional differences, if present, could probably not be detected.

Several workers found that plant tumor tissues could elaborate all of the substances required for growth of normal cells (53–56). Wood (57) reported that 6 of the 7 biosynthetic systems permanently unblocked in crown-gall tumor cells were directly or indirectly activated by specific ions. By varying the levels of 3 salts in the basic medium, he could activate 6 of the essential syntheses. Further, he found that the quantity of total

phospholipids was greater in membranes of the normal cell, and the relative amounts of specific phospholipids varied in the two cell types. Potassium ion could also be absorbed by tumor cells to twice the concentration in normal cells. From these observations, he concluded that when a plant cell transforms to a tumor cell, the membrane properties of the cell are changed so as to permit an increase in solute absorption. This increased permeability of the cell membrane to ions may lead in turn to sequential activation of the biosynthetic systems that become permanently unblocked in the tumor cell.

As noted earlier, plant tissue cells like animal tissue cells may undergo neoplastic conversion during culture *in vitro*. The altered cells can then grow autonomously as tumors when grafted. In the neoplastic conversion the cells acquire the capacity to synthesize auxins and the substances required for growth of normal tissue cells (58-61). It is conceivable that the animal tissue cell could also acquire during long-term culture altered membrane transport and increased capacities for biosynthesis of substances that keep the cell in a state of continued growth and division or prevent the "turning off" of growth and cell division.

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DISCUSSION

Dr. Barski: Dr. Sanford, you mentioned that you were positive about morphologic changes in normal hamster cells, when infected with polyoma virus, forming colonies of a distinct type, or areas of criss-cross growth. You are interpreting it as being due essentially to a proliferative reaction, which means, I understand, that the transformed cells are multiplying in a focus more rapidly than the background nontransformed cells. You mentioned that actually when the transformed malignant cells are compared to separate cultures with normal cells their growth is nearly the same. Now, concerning the cell morphology, does the difference in aspect of normal versus virus-transformed cells continue independently of rapidity of their proliferation?

Dr. Sanford: The morphologic growth pattern induced in hamster embryo cells by polyoma virus may be indistinguishable from that of rapidly growing control cultures

of fibroblast-like cells. In fact, the difference between control and virus-treated fibroblast-like cells is usually noticeable only when the control cultures have a low rate of proliferation. However, there may also be some effect of the virus on cell adhesion to glass and on rate of locomotion, especially in epithelial-like cells. In any case, the cells become less flat, more refractive, and elongated. This alteration may persist.

Dr. White: I have some comments I think are relevant not only to Dr. Sanford's presentation but to that of Dr. Foulds and of Dr. Barski. Dr. Sanford remarked on the significance of the crown-gall studies. The first study of that growth was with crown gall in sunflower. In the sunflower, the requirement was a single substance, one of the auxins. But there are five general classes of substances of that kind—the auxins, kinins, gibberellins, vitamins, and nucleotides—all of which have independent effects on the growth of cells.

What one may call spontaneous alteration has occurred repeatedly in culture in the phenomenon, called "habituation," in which the cells acquire the capacity to make one or another of these substances. It is not always an auxin. It may be a vitamin. This has been demonstrated for biotin; it has been demonstrated for pantothenate and a number of others.

In the case of the tobacco tissues which were first grown, both parents have independent requirements. When crossed, they produce a malignancy, and that malignancy arises from the fact that each parent has a requirement which is supplied by the tissues of the other parent. So that when they are hybridized, each one supplies the requirement of the other. Together, they are autonomous in their growth. Brown's work with tobacco has shown that there may be five independent steps of such acquisition of capacity to synthesize a particular substance. I think this indicates why malignancy in this case is not more common because it involves either the simultaneous mutation toward one or more of these synthetic capacities or the possibility of a chimera in which different cells have mutated for independent requirements.

Dr. Prehn: I would like to emphasize three points.

1) When the biologic potential of culture cells is being assessed, certainly the test animal is fully as important as the cells themselves. For example, we recently observed apparent malignant transformation in mouse cells in as short a time as 6-8 weeks in culture. We believe the reason for this is that we tested these cells after 6-8 weeks in thymectomized irradiated recipients.

2) These transformed cells are highly antigenic, as judged by transplantation studies, apparently fully as antigenic as those cells transformed *in vivo* by methylcholanthrene.

3) Since these cells are antigenic, I believe we now have the means of a fairly critical test as to whether the transformation is actually due to a virus, as Dr. Hayflick proposes. If, indeed, these transformed cells, derived independently in different cultures, prove to cross-react, then the evidence for a virus is very good. But, by analogy with the *in vivo* situation, if we find that they are all independent, then I cannot believe we have activated a different virus in every case.

Dr. Barski: I am sorry to introduce some contradiction to this. We tested our C57BL mouse cell lines for antigenicity by the usual test with animals in which we ligated the tumors obtained with transformed cells, and after their resorption we challenged these animals and intact control mice with a standard inoculation of the same cells. We have exactly the same rate of growth in several experiments with several lines in the control and "immunized" animals. We had to conclude that the cells when tested were not antigenic.

Dr. Prehn: We do have a contradiction, and I am sure we can't resolve it here, but I think that perhaps a step could be made in this direction. It is most important, actually, whether the average run of spontaneously transformed cells is or is not

antigenic. All our work has been done with cells that were in tissue culture for a very short time, and their antigenicity was tested immediately at the conclusion of 6 or 8 weeks. I think this may be of some importance. In this regard, I would like to know, Dr. Barski, how long it was after their point of origin that you tested your cells for their antigenicity.

Dr. Barski: More than 1½ years after starting the culture.

Dr. Sanford: I would like to ask Dr. Prehn if he has examined his cells for leukemia viruses. We found that clone 929-L and our high and low tumor-producing lines from another clone unrelated to strain L were antigenic to mice of the inbred strain of origin. However, all of these lines were found to carry type C particles that may have induced the antigenic alteration.

Dr. Prehn: I am a neophyte, and I will get to it eventually. However, I think that if we find they do not cross-react—and I anticipate this will be the result—then, this almost becomes superfluous.

Dr. G. E. Foley: I am interested in Dr. Sanford's comment on the growth rate of her tumor cells. There is massive support from clinical cancer data that you are correct, at least for the tumors that are important in humans. For example, the adenocarcinomas of the colon that were mentioned have a growth rate measured in days, whereas the normal tissues grow very fast—in 24–48 hours. In connection with chemotherapy in humans, I believe one of the biggest advances in the last few years has been the concept that tumors are very slow growing compared to normal tissues. This has allowed us to use chemotherapeutic agents over prolonged periods that until now have shown no activity in humans and are now showing regressions in human tumors.

Dr. Katsuta: Our two speakers discussed the possibility of a virus in some components of the medium with relation to spontaneous transformation. My comment concerns a transformation that probably occurred in some other way. We have many diploid strains, established strains of parenchymal cells, of rat liver tissue. We cultured these cells in tubes with flattened surfaces for a long period. The tubes were incubated at an angle of 5° in static culture. The medium was renewed twice weekly, very routinely. But the cells were not subcultured for as long as 1 or 2 months.

We found a tremendous change appeared in cell morphology, in cells adhering to the glass surface nearest to the air-liquid interphase, a condition of endomitosis, fragmentation of nuclei, multipolar mitosis, and the atypical condition of the nuclei, etc. These findings attest that mutants are produced at a higher rate at this zone and some of the mutants capable of growing are selected.

We repeated this experiment many times, and we have four mutant lines today. We tried to transfer these cells to rats, but no tumor developed in rats. However, when we transplanted these mutant lines into the hamster cheek pouch, with one certain cell line we got tumors.

Dr. Gey: Someone during the intermission mentioned to me the importance of the size of the inoculum when you are speaking of the ability to make a transplantation. I am delighted that Dr. Foulds also brought up the question of whether an index of neoplasia or malignancy necessarily has any relevance to transplantation.

Dr. Stevenson: As Dr. Hayflick pointed out, the viruses are the only currently available means of reproducibly inducing transformation *in vitro*. Dr. Huebner and his associates have done some recent work which is germane to the point that the subtle interactions between viruses and cells headed some of the great difficulties in resolving the situation. Dr. Huebner's group recently showed with both the Rous and Moloney sarcoma systems that hamster cells can be transformed and yet show no evidence of production in these cells of either infectious virus or any antigens of the neoantigen type. Then, by growing these cells in the presence of either chick embryo fibroblasts or normal mouse embryo fibroblasts, they transferred

this information to the cells. When a helper virus was added—to chicken viruses, the leucosis viruses, or, in the case of the mouse sarcoma, to the murine leukemia viruses—they can then recover the fully infectious sarcoma viruses which transform the hamster cells. This indicates that the data Dr. Sanford and Dr. Hartley obtained on the absence of CF antigen are not in themselves sufficient evidence that these cells do not contain some sort of viral information.

Additionally, Dr. Morris Pollard, at the University of Notre Dame, has shown that, when the six strains of gnotobiotic mice he maintains are irradiated, five of them can give rise to transmissible viral leukemias. In the sixth strain, which is BALB/c, virus-like or leukemia-like particles can be demonstrated in the thymuses of these mice.

We have searched more than 1½ years for strains of mice that do not demonstrate electron microscopic evidence of virus-like particles in the thymus. So far, we have been unable to obtain any such strains.

Finally, another confusing factor is that Dr. Epstein, who was responsible for the isolation of many of the Burkitt lymphoma cell lines, has recently published that he found these herpesvirus-like particles in calf serum.

Therefore, I would like to point out that in any of these studies on transformation, the burden of proof is going to remain on the investigator to show that a virus is not causally linked or associated with the process, and that many of the tools which we now have are either insensitive enough or not sufficiently sharp that we can answer these questions with any degree of certainty.

Dr. Gey: This is one reason I emphasized earlier we need to increase our resolution on many things, since microbiologists years ago indicated that many of our lower forms are rather ubiquitous.

Dr. Sanford: Dr. Stevenson, is your suggestion that we should add some known helper viruses in the hope of extracting from our hamster cells some tumor viruses? It is a system that is a little difficult to test experimentally.

Dr. Stevenson: Yes, and there is no guarantee this system, which has been shown to be operative in these two viruses, is going to be a universal system. At least, it does point out that this subtle integration may be very difficult in cell culture.

Dr. Fjelde: I think we have all been interested for many years in the question concerning the establishment of permanent cell lines, and we still don't know why some cells apparently last forever and others don't. But I would like to point out the study of the adaptation of human tumors to tissue culture in Dr. Alice Moore's department some 14 years ago.

We found that certain patients provided tissue that would do better than others. It seemed that about 20% of the donors provided tissue which was always better, regardless of the condition of the material. From this 20%, three of them became permanent lines. One of them ended up as the famous line HEP-2.

I will have to investigate, I think, the three lines we have carried for 13 years. It might be worthwhile to investigate why some people provide cells that grow better than those of other persons. You might find enzymes or metabolic changes in their cells that would be very useful.

Dr. de Bruyn: Just a few remarks in regard to the beautiful presentation of Dr. Sanford. At the Netherlands Cancer Institute, Amsterdam, tumors and cell lines were studied that derived from a transplantable lymphosarcoma, which originated in 1935 in a female mouse, a hybrid between the C57BL strain of Little and the DBA strain of Murray and Little. From one subcutaneous tumor, cell lines containing mesenchymal cells and lymphoblasts together, and lines containing either fibroblast-like cells or lymphoblasts alone, have been maintained in continuous culture for periods of 29, 29, and 21 years, respectively. The lymphoblasts which are the tumor-producing cells have a very stable chromosome number (40, 41), whereas the chromosome constitution of the fibroblast-like cells has much changed. The

changes were observed in cell lines which at that time could not produce tumors when injected subcutaneously in newborn or in young adult, untreated mice. In accordance with results reported by Dr. Sanford, these findings seem to prove that heteroploid, established cell lines do not necessarily have malignant properties. In comparison with the results of other investigators, it took a long time (10 years) before the fibroblast-like cell lines occasionally produced tumors in newborn and young adult mice. The reason for this delay in so-called "neoplastic transformation" could perhaps be that other methods and nutritional fluids were used. In this respect, it is interesting that in experiments reported by Dr. Sanford the fetal calf serum seems to influence the time of this "neoplastic transformation." Further research in this line should be done.

Dr. Johnsson-Hegyesi: I have three points to make.

The first is a question addressed to Dr. Sanford: The experiments you reported show that fetal calf serum rather than horse serum in the culture medium delays the neoplastic transformation of mouse cells *in vitro*. Have you tried comparing serum from the same species prepared from animals of different age groups? It is possible that the observed difference is related to the age of the animal from which the serum was prepared.

My reason for asking this question has to do with some experiments by Paul Weiss and his pupil J. W. Saunders, Jr.,¹ on chick embryos. They found that the growth of epidermal cells into a smooth monolayer rather than in a piled-up fashion was dependent on the emergence in the embryo of an unknown factor which became active at age 10 days, *i.e.*, about halfway through embryonal development. The appearance of this factor coincided with the period in which the major endocrine systems in the chick start to function. When a wound was made on the skin of a chick embryo before age 10 days, the skin gap remained open in spite of a piling up of epithelial proliferating cells at the edges of the wound. Exactly at the age 10 days, the piled-up cells started wandering out over the wound surface in a monolayer fashion. Since one of your criteria for transformation was a piling up of the cells in monolayer cultures, the delayed emergence of transformation in the presence of fetal calf serum might be related to the presence of more of a "monolayering factor" in the serum from the calf fetus than in the serum from the adult horse.

My second point refers to your statement regarding the current assumption that all continuous cell lines have acquired the properties of cancer cells and can no longer be distinguished as being normal or malignant in origin. Your data suggested that this assumption is incorrect. I would like to report to the Conference on the finding in human urine of a factor which distinguishes between heteroploid cell lines of normal origin and heteroploid cell lines of malignant origin.² It has a molecular weight of around 600, possesses a biologically active phosphate group, and induces polarization and directional growth of 4 malignant cell lines, but not of 5 normal ones (fig. 1).

The third point is in response to Dr. Gey's introductory comment that there is a tendency to think of cells *in vitro* as being synonymous with cells *on glass*, and that this might be unfortunate. Recent experiments at Battelle Institute, Columbus, Ohio, have shown that opaque metal surfaces, such as stainless-steel 304, 347, and gold, are favorable surfaces for the growth of mammalian cells, and that these living cultures can be photographed in color by means of reflected light interference microscopy against their opaque background (fig. 2).

Dr. Gey: We now have a special request from Dr. Heidelberger to present briefly some material of his.

¹ WEISS, P., and SAUNDERS, J. W., JR.: *Anat Rec* 108: 93, 1950.

² JOHNSSON, R., and HEGYELI, A.: *Proc Nat Acad Sci USA* 54: 1375-1378, 1965.

Dr. Heidelberger: For 20 years we have been trying to find the biological and molecular mechanisms of hydrocarbon carcinogenesis *in vivo* with depressingly few results. It seems obvious an *in vitro* system would be preferable. This is a brief progress report.

It seemed that the system with mouse prostate originated by Dr. Ilse Lasnitzki would be useful for this purpose, and I had the privilege of studying in her laboratory.

When young adult mouse prostates are cultivated in organ culture without the addition of hydrocarbons, they usually maintain a differentiated appearance. However, if a few $\mu\text{g/ml}$ of methylcholanthrene (MCA) are added, we get massive hyperplasia, pleomorphic nuclei, anaplasia, and multipolar abnormal mitoses. Some pathologists have thought that these sections look malignant.

To demonstrate this, Dr. Roeller and I spent $3\frac{1}{2}$ years unsuccessfully putting these pieces into C3H mice under many different conditions and only obtained one spontaneous tumor in the lot. We, therefore, decided to start with these mouse prostates, and treated them in organ culture for 3 weeks in minimal Eagle's medium with horse serum. The controls received no hydrocarbon. The carcinogen-treated cells were treated for 1 week with MCA in organ culture, 2 weeks in the BME medium, and then were dispersed with pronase and put into cell culture.

Text-figure 1 indicates what we found in terms of cell numbers. The dotted lines are the controls that failed to grow.

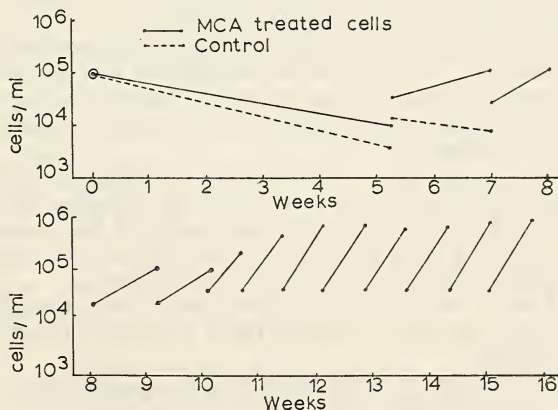
In 20 consecutive experiments in which we have supplemented BME with all different types of media, we have never succeeded in getting cell lines from these control prostates that have been grown in organ culture without carcinogen treatment. Therefore, we have no evidence of spontaneous transformation.

The carcinogen-treated cultures eventually in 3-5 weeks develop foci of cells that grow, and by the 10th week, they grow rapidly.

In the experiment illustrated here, a cell line has been obtained that has grown continuously for 10 months at an approximately constant and rapid growth rate.

Figure 1 shows the appearance of these cells in phase contrast on the dish in monolayer culture. They appear to be epithelial.

Figure 2 demonstrates that, when these cells are allowed to grow longer in the monolayers, they pile up on top of each other and form a randomly oriented criss-



TEXT-FIGURE 1.—History of cell cultures derived from organ cultures of mouse prostate (see text for description). This culture has been maintained for more than 9 months with doubling times comparable to those obtained from 12 weeks on. Each line on the graph represents a subculture after 5 weeks.

cross pattern as shown in the stained preparation. These cells have a high acid phosphatase content, which is characteristic of prostate, and are resistant to the toxicity of carcinogenic hydrocarbons. These transformed cells produce tumors when one million cells are inoculated into adult C3H mice without previous conditioning with X-ray or cortisone treatment. Most of these tumors are fibrosarcomas. But, in the case of methylcholanthrene, we have obtained two anaplastic carcinomas. These cells have been kept in culture for fairly long periods, and we are now trying to obtain tumor induction in shorter periods.

Figure 3 shows the histological appearance of one of the fibrosarcomas. These tumors are all readily transplantable, once they are induced in the mice with these primary explants from the cell culture.

Figure 4 shows the histology of the tumor that has been diagnosed by pathologists as an anaplastic carcinoma.

These are the criteria which we believe establish that we have produced malignant cell transformations *in vitro* with carcinogenic hydrocarbons in this system which, as you recognize, is quite different from that of Berwald and Sachs.

1) The normal prostate cells all die, whereas the carcinogen-treated cells form permanent lines.

2) The treated cells pile up in a random orientation.

3) The treated cells are much less susceptible to the toxicity of carcinogenic hydrocarbons than normal cells. As Dr. Prehn and others realize, this is one of the characteristics of malignancy.

4) Aneuploid cells and multipolar mitoses are seen in the cultures.

5) The metaphase-prophase ratio is high in mitotic cells, which is considered to be characteristic of malignancy. I don't have time to go into that.

6) The treated cells are rich in acid phosphatase.

7) On implantation of one million cells into adult unconditioned mice, malignant transplantable tumors are produced.

I merely present the facts and do not have time to go into interpretation.

Dr. Barski: Are your cell lines antigenic?

Dr. Heidelberg: This is one of the many things we have yet to do. We hope to study this very soon.

Dr. Sanford: I would feel better about your results if you had been able to grow your controls. As you know, almost every strain of cells that we have grown from C3H mice by this period in culture would be neoplastic.

Dr. Heidelberg: These are adult epithelial cells. We have tried very hard to grow them. One might say there are two transformations that take place. The first is the transformation by the hydrocarbon to produce a permanent cell line. A second transformation to malignancy, superimposed on the first, may or may not be spontaneous. We are trying to find this out.

Dr. Hayflick: As I pointed out before, this proposal that viruses may be involved in the spontaneous malignancy *in vitro* is merely an extension of what we can reproducibly do with known viruses. I was impressed with the presentation of Dr. Heidelberg. It was the kind of evidence that many of us have been awaiting for quite some time and wondering why it was so long in coming.

Dr. Sanford: I would like to ask Dr. Heidelberg a question which Dr. Gey has been wondering about, too. How did you get rid of the 5 $\mu\text{g}/\text{ml}$ of MCA with which these cells were treated?

Dr. Heidelberg: We got rid of this by washing the organ cultures very carefully and transferring them to another organ culture medium without the carcinogen for a period of 2 weeks.

If there had been sufficient carcinogen left in these pieces to induce tumors, we would have obtained some tumors, presumably, during those 3½ years we implanted organ-culture pieces into mice. We failed completely. We have some experiments

going, using autoradiography with tritiated hydrocarbons. We can find evidence of very high specific activity, but no detectable grains in the cells after they have gone into cell culture.

If I may make one comment, we are trying to show that viruses are not involved. We have ground up our organ-culture pieces and cell-culture pieces that are transformed, made very concentrated cell-free extracts, and put these in a variety of cells, and have not seen any cytopathic effect. Sera of the mice in which the tumors have been induced do not contain polyoma antigens or antibodies. They have been preserved and sent to the National Cancer Institute to be examined for leukemia viruses.

These experiments, of course, don't prove anything. We have sections waiting to be examined in the electron microscope to look for particles.

Dr. Sanford: I still feel, however, that until you have controls in this experiment, the results do not mean too much, because, by 10 months of culture, strains of cells we have grown from the C3H mouse have become neoplastic. This applies to adult epithelial tissues like those from the parotid.

Dr. Heidelberger: In the organ culture going into cell culture, we have tried very hard and have failed to get these cells to grow. If we start out with fresh mouse prostate and put this directly into cell culture, we get a mixed population of fibroblasts and epithelial cells. The epithelial cells take over and grow fairly rapidly for about 3 or 4 subcultures. But in spite of many experiments, these also die out.

Dr. Sanford: What medium are you using?

Dr. Heidelberger: We are using a variety of media. We are using BME plus 20% fetal calf serum, and we use fetal calf serum throughout. We have used 199 and NCTC 109. We have added embryo extract. We have done all the tricks we could think of to make these cells grow. I admit it is embarrassing not to have controls.

Dr. Sanford: However, one cannot conclude that the chemical carcinogen has induced the malignant transformation, if from all available evidence, cell lines after this period in culture can undergo malignant conversion without carcinogen.

Dr. Heidelberger: We know we have never failed to get them to grow with hydrocarbons in six successive experiments. So at least the hydrocarbon makes them grow under conditions where we cannot get the untreated cells to grow. The hydrocarbon, we feel, has definitely done something. Whether the acquisition of malignancy, as defined by transplantability later on, is spontaneous or not, we don't know. But now we are setting out systematically to inject cells into mice at every single transplant. We believe we will probably get tumors fairly early. But we will have to find out.

Dr. Kniazeff: My question is in reference to the use of fetal calf serum. Contrary to what most of the texts on immunology and physiology state, which is that transference of γ -globulin in the bovine species does not take place through the placenta, we have found in a recent study that 40% of individually harvested fetal calf serum specimens contain γ -globulin. We have also shown that some of these will neutralize specifically some of the bovine viruses. The levels of the γ -globulin, if not sufficient to completely neutralize a large concentration of virus, are quite adequate to establish a latent infection if the initial virus infection is low. This is a point for you to consider in your studies.

Dr. Freeman: There is another example of a component of the medium playing a role in apparent neoplastic conversion. In this case, it is not spontaneous transformation but transformation of whole rat embryo cells with adenovirus-12. The component is chemically defined as calcium, and the rate of transformation of whole rat embryo cells is significantly affected by the concentration of calcium in the medium.

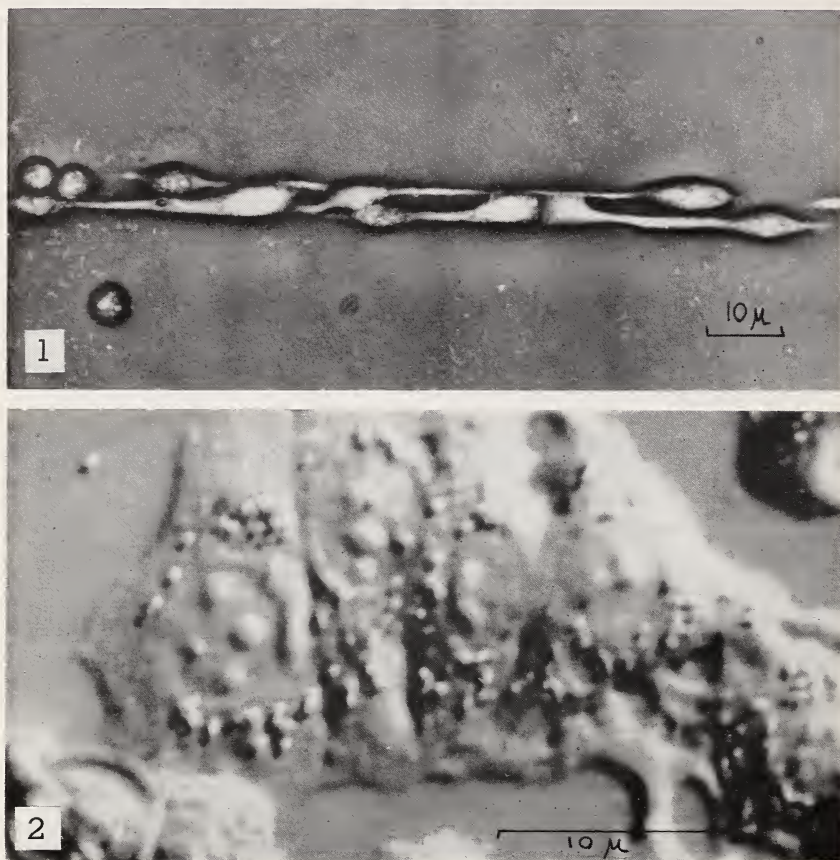


FIGURE 1.—Detail of living, unstained 72-hour-old KB culture, exposed to directin for 48 hours. Extract prepared from human urine by Dr. Andrew Hegyeli, Battelle Institute, Columbus, Ohio.

FIGURE 2.—Detail of living, unstained 72-hour-old KB culture, growing directly on stainless-steel opaque coverslip. Photographed by reflected light interference optics.

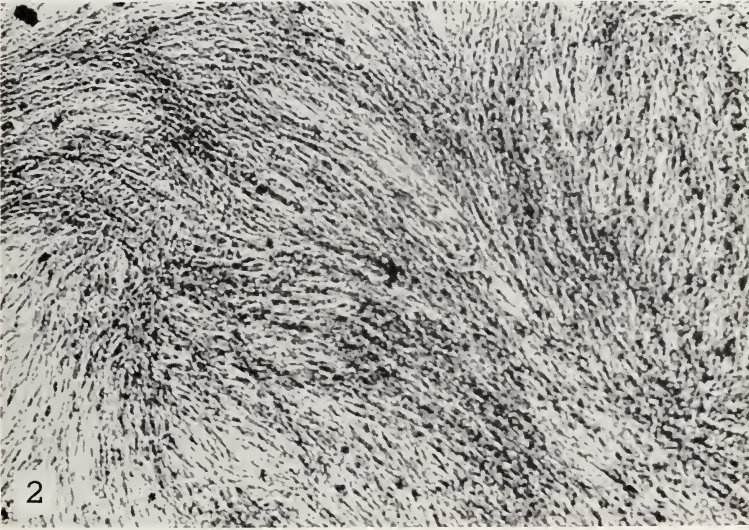
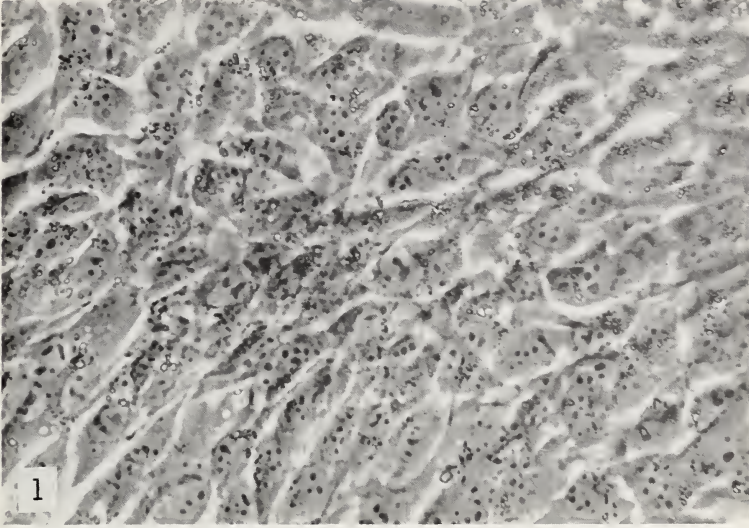


FIGURE 1.—Epithelium-like cells from methylcholanthrene (MCA) treated organ culture after 113 days in cell culture. Phase-contrast photomicrograph. $\times 600$

FIGURE 2.—MCA-treated culture after 113 days in cell culture. The petri dish was fixed in methanol and stained with Giemsa. Photomicrograph. $\times 240$

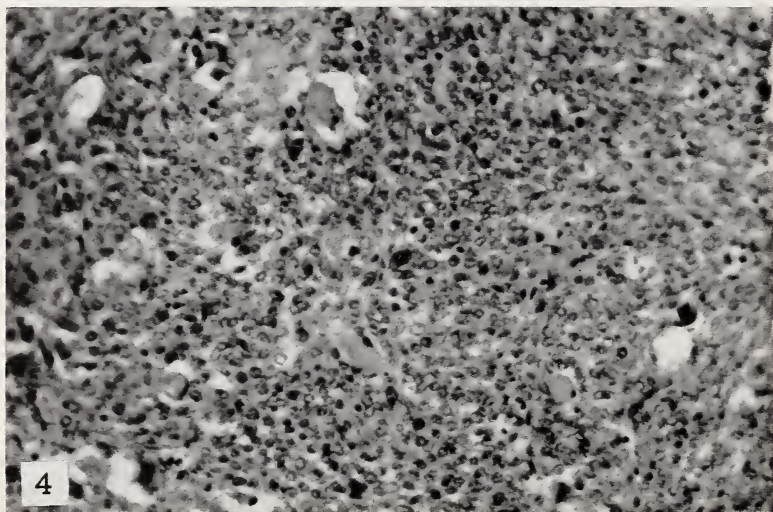
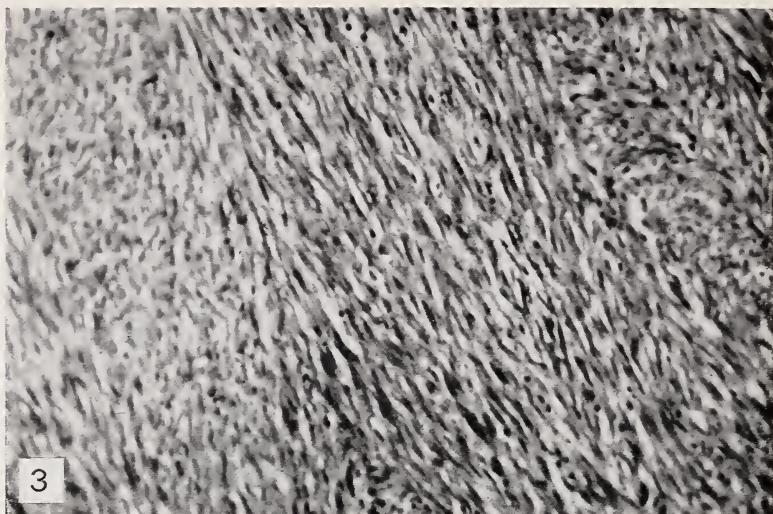


FIGURE 3.—Fibrosarcoma induced in male C3H mouse by implantation of 1×10^6 cells transformed *in vitro* with methylcholanthrene (MCA). This was fixed in formalin and stained with hematoxylin and eosin. Photomicrograph. $\times 210$

FIGURE 4.—Anaplastic carcinoma induced in male C3H mouse by implantation of 1×10^6 cells transformed *in vitro* with MCA. This was fixed in formalin and stained with hematoxylin and eosin. Photomicrograph. $\times 210$

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